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Enzymatic carotenoid cleavage in star fruit (*Averrhoa carambola*)

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Abstract

This paper presents the first description of an enzyme fraction exhibiting carotenoid cleavage activity isolated from fruit skin of *Averrhoa carambola*. Partial purification of the enzyme could be achieved by acetone precipitation, ultrafiltration (300 kDa, 50 kDa), isoelectric focusing (pH 3–10) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%). In this way, an enzymatically active protein fraction was obtained, consisting of four proteins in the molecular weight range of between 12 and 90 kDa. Using β-carotene as substrate, the enzyme activity was detected spectrophotometrically at 505 nm. The main reaction product, detected by GC analysis, was β-ionone. This proves that the isolated enzymes are closely related to aroma metabolism and release of star fruit. The time constant of the reaction was 16.6 min, the Michaelis Constant $K_m = 3.6$ μmol l^{-1} and the maximum velocity $V_{max} = 10.5 \times 10^{-3}$ μmol l^{-1} s^{-1} $mg_{(Protein)}{}^{-1}$. The optimum temperature was 45 °C.

Keywords: *Averrhoa carambola*; Fruit; Carotenoids; Oxidative cleavage; β-Ionone

1. Introduction

Volatile carotenoid breakdown products are long known as important flavor compounds, in various fruit (Winterhalter and Rouseff, 2001a). The flavor of star fruit (*Averrhoa carambola*) is strongly influenced by these carotenoid derived compounds such as β-ionone (Winterhalter and Schreier, 1995). However, until now the biochemical pathways giving rise to those flavor compounds are not fully elucidated.

Apart from chemical oxidative degradation of carotenoids, enzymatic oxidative cleavage is seen as an important flavor formation pathway (Enzell, 1985; Wahlberg and Eklund, 1998; Winterhalter and Rouseff, 2001b; Winterhalter and Schreier, 1988). However, whereas enzymatic cleavage of carotenoids in animal tissues is well known (Glover et al., 1960; Lakshman and Mychkovsky, 1989; von Lintig and Vogt, 2000; Wolf, 2001; Wyss et al., 2000), the number of reports giving evidence for similar reactions in plant tissues is still limited (Fleischmann et al., 2001, 2002; Schwartz et al., 1997, 2001). With the exception of enzymes specifically degrading 9-*cis*-neoxanthin to abscisic acid (Schwartz et al., 1997), there is only information about carotenoid cleavage enzymes in *Arabidopsis thaliana* (Schwartz et al., 2001) and in quince (Fleischmann et al., 2002), both with C_{13} and C_{14} compounds as most likely reaction products.

In the present paper, we describe the partial purification of an enzyme fraction with carotenoid cleavage activity from star fruit (*Averrhoa carambola*, Oxalidaceae) skin. This cytosolic activity is characterized by its kinetic parameters (V_{max}, K_m, time constant, temperature dependence) and its main reaction product.

2. Results and discussion

2.1. Isolation and characterization of star fruit carotenoid cleavage enzymes

Despite the highly lipophilic nature of their substrate, carotenases are cytosolic proteins (Fleischmann et al., 2001; Schwartz et al., 2001; von Lintig and Vogt, 2000). Consequently, they are only found in water soluble fractions of star fruit skins, whereas no activity could be traced in other fractions or fruit tissues. Importantly, only extracts from fully ripened star fruit showed carotenoid

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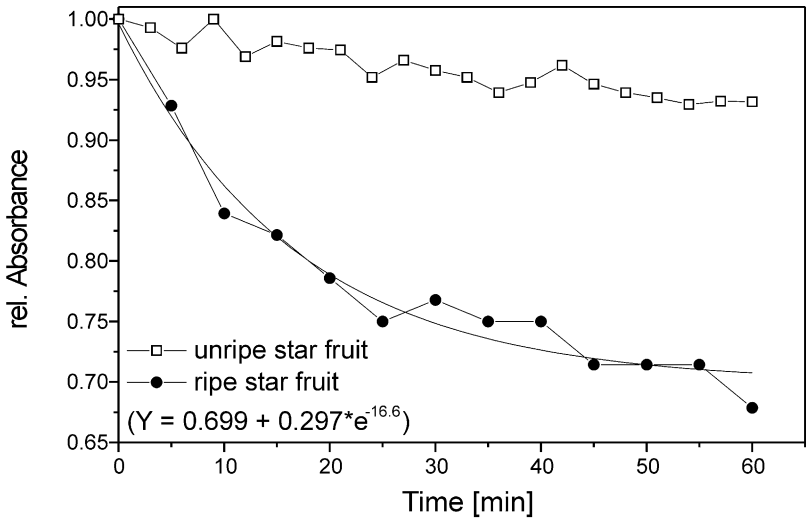

Fig. 1. Kinetics of β-carotene degradation by enzymes isolated from unripe and fully ripened star fruit (*Averrhoa carambola*) (40 °C, pH = 7.0, 2.48 μmol/l initial β-carotene concentration, 24.1 μg/ml protein concentration). For ripe star fruit, an exponential regression curve was calculated (time constant = 16.6 min).

cleavage activity (Fig. 1). Extracts from unripe star fruit do not show any measurable enzymatic carotenoid cleavage activity. The slow decline in β-carotene concentration shown in Fig. 1 is undistinguishable from chemical degradation at the same conditions. After isoelectric focusing, enzyme activity was concentrated in a single peak (Fig. 2). The peak fractions were pooled as partially purified carotenoid cleavage enzyme. After SDS PAGE and silver staining, four protein bands were detected in the enzyme solution (Fig. 3). The molecular weight of the proteins was calculated from the calibration curve of the standard proteins (R_f value vs. mol. mass) used for the SDS PAGEs. Three bands between 50 and 90 kDa and one protein band at 12 kDa are present at the SDS PAGE shown in Fig. 3. Together with the fact that the two bands around 90 kDa and the band at 12 kDa are present in inactive protein fractions as well (lane 2, Fig. 3), it is most likely that the 50 kDa band represents the enzyme responsible for the carotenoid cleavage described here. Furthermore, the molecular weight of this protein is close to that reported for carotenases isolated from other sources (66 kDa). Carotenoid cleavage enzymes isolated from *Arabidopsis thaliana* (Schwartz et al., 2001), as well as enzymes isolated from chicken (related to retinal formation) (Wyss et al., 2000) belong to this group. Additionally, VP14 type 9-*cis*-epoxy-carotenoid cleavage enzymes, which are involved in abscisic acid formation in species like

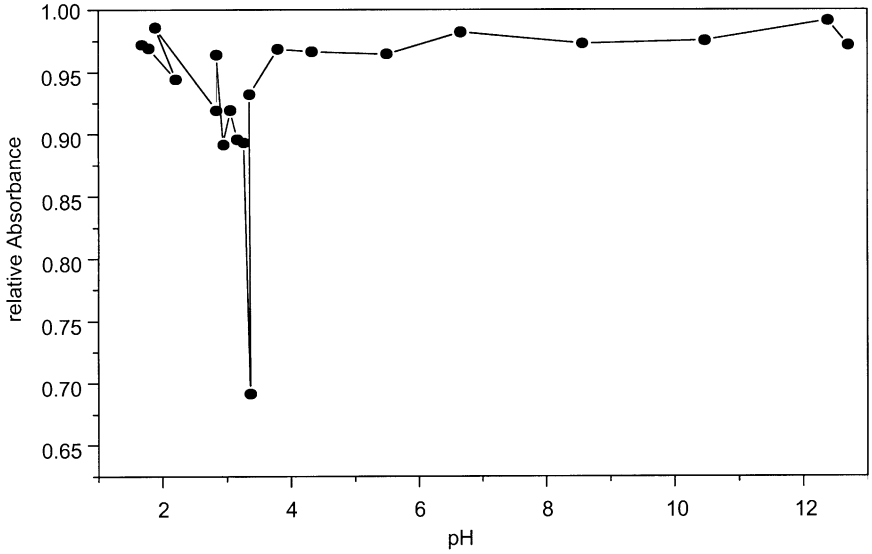

Fig. 2. Activity pattern of the enzyme after preparative isoelectric focusing. The peak shows the approximate value of the isoelectric point (pH = 3.6) of the enzyme.

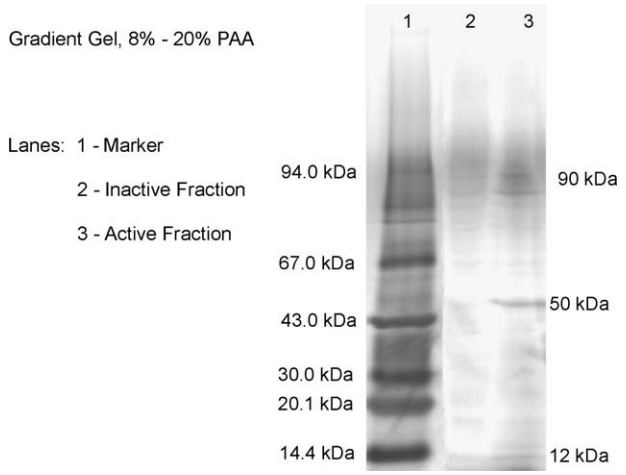

Fig. 3. Polyacrylamide gel electrophoresis of the partially purified enzyme.

avocado (*Persea americana*) (Chernys and Zeevaart, 2000), bean (*Phaseolus vulgaris*) (Qin and Zeevaart, 1999), and again *Arabidopsis thaliana* (Schwartz et al., 1997), are known to have very similar molecular masses.

2.2. Kinetic properties

The time constant of the reaction was calculated by non linear regression of the β-carotene degradation time course (Fig. 1). The value obtained was 16.6 min. Fig. 1 shows that the time course of the carotenoid cleavage reaction could be fitted to an exponential function, typical for first order reactions. Therefore, under our experimental conditions, the influence of other substrates than β-carotene (i.e. oxygen) is negligible. Consequently, it was possible to use first order Michaelis–Menten kinetics for a description of the carotenoid cleavage activity.

The kinetic parameters (V_{max} and K_m) of the carotenoid cleavage enzymes isolated from star fruit were studied at pH 7.0 and 23 °C. K_m and V_{max} were calculated by linear regression ($R=0.99$) of the enzyme's Lineweaver–Burk plot, using β-carotene as substrate (Fig. 4). The values obtained ($V_{max}=10.5\times10^{-3}$ μmol l^{-1} s^{-1} $mg_{(Protein)}{}^{-1}$; $K_m=3.6$ μmol l^{-1}) are clearly different from values determined for plant carotenases isolated from other sources (Fleischmann et al., 2001, 2002). The K_m of the star fruit carotenases described here is approximately 4 times lower than the one reported for quince carotenases, whereas the V_{max} is about 10 times higher compared with quince (Fleischmann et al., 2002). Therefore carotenases isolated from star fruit skin show clearly higher affinity to the substrate β-carotene (K_m) and also a higher turnover efficiency (V_{max}) compared to carotenases obtained from quince, supporting the idea of at least two different types of carotenoid cleaving enzymes in fruit tissues.

The temperature profile of star fruit carotenases shows an increase of enzyme activity until the maximum temperature measured (Fig. 5). Despite the increasing non-enzymatic β-carotene degradation at higher temperatures (Fig. 5), the data show that the optimal operating temperature of this enzyme is approximately 45 °C. This is not surprising, because of the similar optimum temperature of already known fruit carotenases found in the same range (Fleischmann et al., 2001). The data used for the determination of the optimum temperature were subsequently used for the calculation of the activation energy E_a. Fig. 6 shows an Arrhenius plot of these data in semi-logarithmic form ($\ln v \rightarrow 1/T$). The slope of the plot shown in Fig. 6 (calculated from the equation of the regression curve) was used to calculate the activation energy $E_a=101.5$ kJ mol^{-1} of the cleavage reaction. This value is higher than the typical activation energy levels of enzymes (Bisswanger, 1979). For water soluble enzyme specifically oxidizing a highly lipophilic substrate like β-carotene, enzymes and substrate molecules (usually provided in micellar form) should reside in different compartments of the incubation mixture. Therefore it is not surprising to find higher differences in energy levels (and therefore activation energy) between single substrate and enzyme molecules on the one hand and the enzyme-substrate complexes on the other.

The optimum pH of β-carotene degradation activity could be detected at pH 8.5 (Fig. 7). At pH values lower than 7, Fig. 7 shows an increase in activity again. But the blank data show that this is caused only by an increase in non-enzymatic chemical degradation. These results differ from those reported for enzymes derived from quince, which did show activity changes dependent on the pH of the incubation mixtures (Fleischmann et al., 2002). In order to further investigate this discrepancy, studies on the influence of the different origins of the plants (quince from temperate European, starfruit from tropical climate) are currently planned.

2.3. Reaction products

Finally, GC experiments proved that the major volatile reaction product isolated from incubations of β-carotene with isolated star fruit carotenoid cleavage enzymes is β-ionone. GC analyses of reaction products extracted from active incubation mixtures as described in 3.5 (attached to an SPME fiber) show a single peak at 35.8 min. Co-injection experiments of isolated reaction products together with standard β-ionone also resulted in a single peak (35.9 min) under the same running conditions in GC. Therefore we can conclude that biooxidative carotenoid cleavage enzymes in star fruit skins exclusively cleave β-carotene eccentrically at the 9–10 (9′–10′) double bond with β-ionone as the dominant volatile reaction product. Together with their dependence on the ripening state of the fruit, these results prove the importance of carotenoid cleavage enzymes for aroma

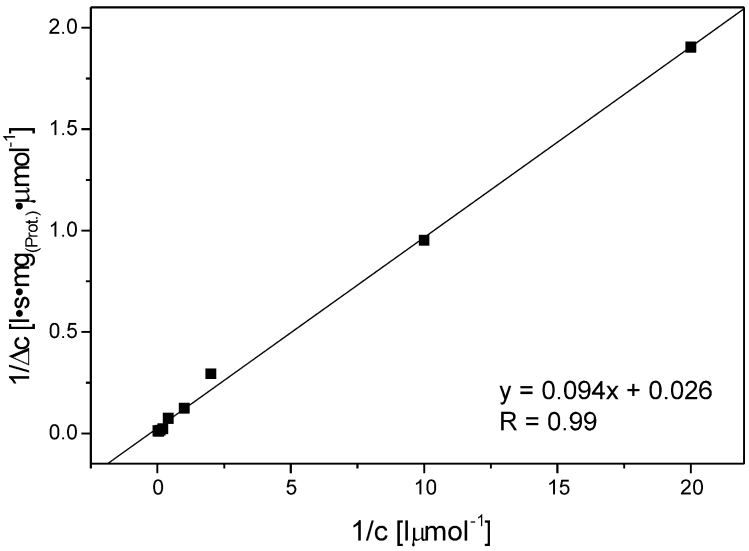

Fig. 4. Lineweaver–Burk plot with regression curve and calculated kinetic key constants of star fruit carotenoid cleavage enzyme.

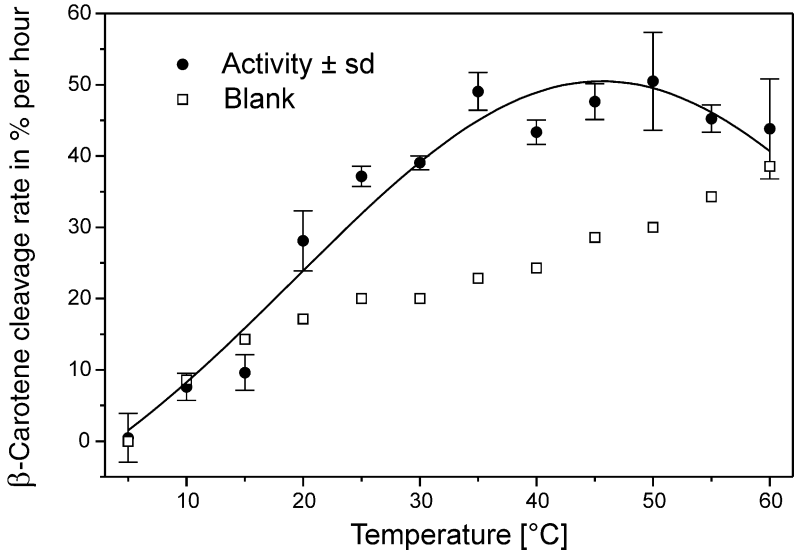

Fig. 5. Temperature dependence of star fruit cleavage enzymes. At 45 °C, we could trace the highest enzyme activity. sd = Standard deviation.

production and metabolism in ripening and ripe star fruit. However, until now no second reaction product, i.e. carotenoid fragment, could be detected, what is mainly due our methodology (GC analysis). Further experiments are currently undertaken in order to identify non-volatile reaction products.

3. Experimental

3.1. Plant material

Star fruit (*Averrhoa carambola*) has been obtained from a local fruit market in Shizuoka, Japan. In order to isolate enzymatically active protein fractions, it was necessary to store the fruit at room temperatures (24 °C) until fully ripened (at least 2 weeks). This ripening period was needed to induce formation of intense flavor as well as relevant amounts of carotenoid cleavage enzymes inside the fruit-skin. During ripening, the green, almost scentless fruit changed to intensively yellow color and fruity, ionone-like aroma.

3.2. Chemicals

β-Carotene was purchased from Sigma (Japan). Biolyte 3-10 ampholytes used for isoelectric focusing were obtained from BioRad (Hercules CA), Tris–HCl, $MgCl_2$, KCl, Tween 40, sodium dodecyl sulfate (SDS), acrylamide, and bisacrylamide were all purchased from Wako, Japan. All other reagents were at least of analytical grade.

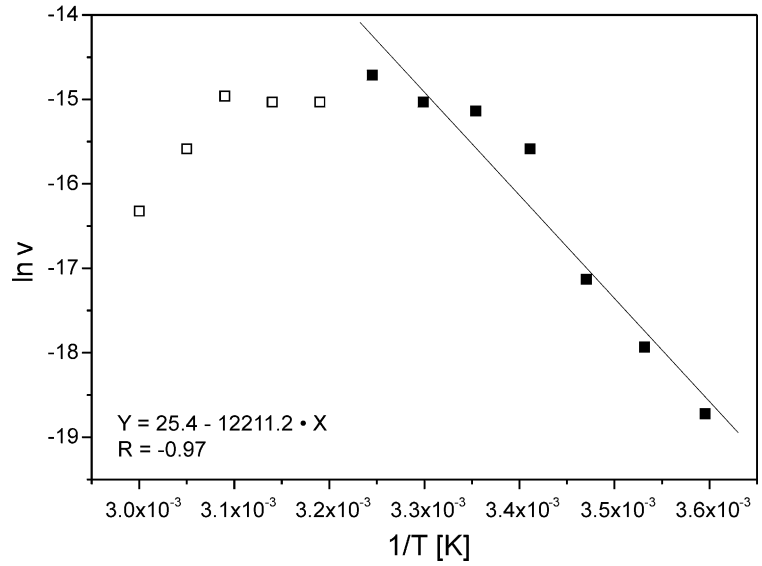

Fig. 6. Arrhenius plot of the star fruit cleavage enzymes. The activation energy of the cleavage reaction $E_a = 101.5$ kJ mol^{-1} could be determined from the slope of the regression curve. The regression was performed for the black (filled) data points.

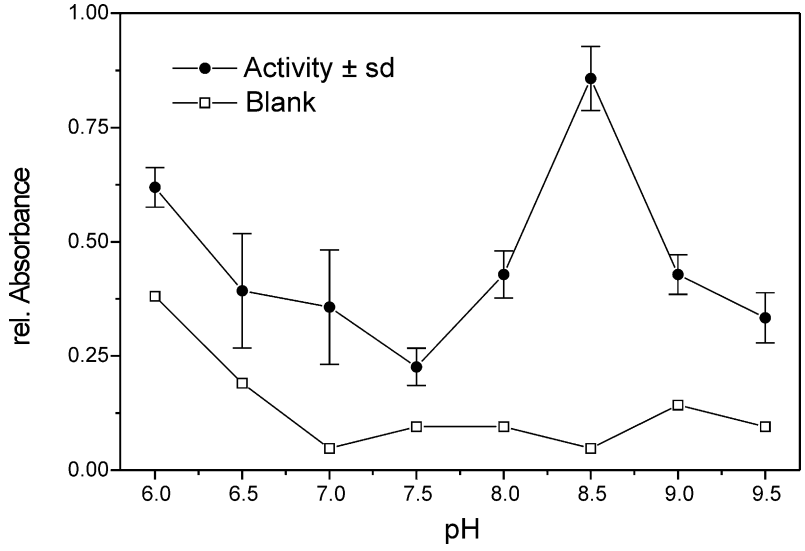

Fig. 7. pH dependence of star fruit carotenoid cleavage enzymes. The optimal pH could be found at pH = 8.5. sd = Standard deviation.

3.3. Enzyme isolation

After removing the outer wax layer of fully ripened star fruit (1.0 kg) with *n*-hexane (10 s), the peelings were homogenized in sample buffer (125 mmol/l KCl, 5 mmol/l $MgCl_2$, 50 mmol/l Tris, pH 6.8, 100 ml per 100 g of fresh fruit skin) using an ultra Waring blender (120 s). The homogenates were centrifuged at 2000 *g* for 10 min. The resulting pellet was discarded and the supernatant subjected to an acetone precipitation (87%, 3 h, 10.0 °C). The resulting precipitate was dissolved in sample buffer and subjected to a preparative isoelectric focusing (Bio-Rad Rotofor, pH 3–10, 12 W, 7 h, 4 °C,). All protein fractions resulting from isoelectric focusing were screened for carotenoid cleavage activity. In order to remove the ampholytes needed for isoelectric focusing, the active fractions were ultrafiltrated at 50 kDa. The protein fractions resulting from this step were stored in sample buffer (1 °C) until used as enzyme extract. All workup steps were carried out at 4 °C.

3.4. Enzyme assay and kinetic methods

Carotenoid cleavage activity was measured spectrophotometrically with β-carotene as substrate. β-Carotene (0.1 mg/ml) was dissolved in water using Tween 40 (10% in stock solution) as emulgator. The reaction was carried out in microcuvettes (final volume 375 μl) in the spectrophotometer, using an initial β-carotene concentration of 2.48 μmol/l in the reaction mixture. For

measuring the Lineweaver-Burk plot the assay was performed at 9 different substrate concentrations between 0.05 and 50 μmol/l. The enzymatic reaction was measured against β-carotene control solutions under the same reaction conditions, but without protein. Molecular oxygen, the only co-substrate needed (and used) for the carotenoid degradation reaction, was provided in abundance using air saturated buffer solutions. The carotenoid cleavage reactions were monitored continuously at 505 nm. At this wavelength, it was possible to trace the β-carotene cleavage uninfluenced from the spectral absorption of resulting reaction products.

The temperature dependency of enzyme activity was measured similar to the method described above. However, here only the reaction cuvettes contained β-carotene. The reference cuvettes contained β-carotene free, otherwise identical solutions. For the control measurements, the enzymes were heat deactivated (90 °C, 10 min) prior to the incubations. No difference in β-carotene degradation could be detected between protein free incubation solutions and incubations containing deactivated enzymes. The activation energy of the cleavage reaction was calculated using the logarithmic form of the Arrhenius equation:

$$\ln k_{\mathrm{cat}} = \ln k_0 - \frac{E_{\mathrm{a}}}{R \cdot T}$$

At the beginning of the incubation period, k_{cat} can be regarded as almost independent of the reverse reaction. Consequently, for enzyme catalyzed reactions like the cleavage of β-carotene, the ln k_{cat} can be replaced by the logarithm of reaction speed, ln v. The slope of a plot ln $v \rightarrow 1/T$, (multiplied by $-R$) exhibits the activation energy E_{a} in J mol^{-1}. The left part of the data set (Fig. 6) was not included into the fit in order to avoid possible influence of enzyme deactivation or heat denaturation on the calculation of E_{a}. Finally it is necessary to note that the E_{a} value calculated here is underestimating the real activation energy of this carotenase. This was unavoidable because of the extremely high absorbance of β-carotene at saturating conditions (50 μmol/l; $A > 3$).

3.5. Determination of proteins and reaction products

The protein content was measured according to the Biuret method using BSA as standard protein.

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a Mini Protein Cell (Wako Corp.) using commercially available 7.5% polyacrylamide gels (Wako Corp.). The gels were stained with a commercially available silver stain kit (Wako Corp. Japan). All runs were carried out under constant voltage conditions (90 V) at room temperature.

The reaction products were detected by gas chromatography (Column: GL Sciences TC-WAX, 0.25 mm, 30 m; temperature profile: 35–230 °C, 3 °C/min; injection temperature: 250 °C; carrier gas: N_2, 1.0 ml min) after extraction from the reaction mixture. For extracting volatile norisoprenoids from the incubated enzyme solutions, Bio-Beads SM-2 (Bio Rad) were added directly after the end of incubation. After 3 h, the Bio-Beads were removed from the incubation mixture and washed twice with Milli-Q for 10 s. The beads were then transferred into a stirred methanol solution (20 ml, 2 h, room temperature) to redissolve the volatiles. This step was repeated three times. The methanol fractions were combined and concentrated by a Vigreux column to a final volume of 500 μl. Finally, the reaction products were transferred to SPME fibers (Polymethylsiloxane, 100 μm; 10 min incubation time, 23 °C), which were directly used for the GC experiments.

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References

Bisswanger, H., 1979. Theorie und Methoden der Enzymkinetik. Verlag Chemie, Weinhein, Basel, Deerfield Beach.

Chernys, J.T., Zeevaart, J.A.D., 2000. Characterization of the 9-*cis*-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in avocado. Plant Physiol. 124, 343–353.

Enzell, C., 1985. Biodegradation of carotenoids—an important route to aroma compounds. Pure and Appl. Chem. 57, 693–700.

Fleischmann, P., Lutz-Roeder, A., Winterhalter, P., Watanabe, N., 2001. Carotenoid cleavage enzymes in animals and plants. In: Winterhalter, P., Rouseff, R. (Eds.), Carotenoid-Derived Aroma Compounds. ACS Symp. Ser. 802, Vol. 219. American Chemical Society, Washington, DC, pp. 76–88.

Fleischmann, P., Studer, K., Winterhalter, P., 2002. Partial purification and kinetic characterization of a carotenoid cleavage enzyme from quince fruit (*Cydonia oblonga*). J. Agric. Food Chem. 50, 1677–1680.

Glover, J., Deretic, D., Papermaster, D.S., 1960. The conversion of β-carotene into vitamin A. Vitamins and Hormones 18, 371–386.

Lakshman, M.R., Mychkovsky, I., 1989. Enzymatic conversion of all-*trans*-β-carotene to retinal by a cytosolic enzyme from rabbit and rat intestinal mucosa. Proc. Natl. Acad. Sci. USA 86, 9124–9128.

Qin, X.Q., Zeevaart, J.A.D., 1999. The 9-*cis*-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proc. Natl. Acad. Sci. USA 96, 15354–15361.

Schwartz, S.H., Qin, X.Q., Zeevaart, J.A.D., 2001. Characterization of a novel carotenoid cleavage dioxygenase from plants. J. Biol. Chem. 276, 25208–25211.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart, J.A.D., McCarty, D.R., 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science 276, 1872–1874.

von Lintig, J., Vogt, K., 2000. Filling the gap in vitamin A research—molecular identification of an enzyme cleaving beta-carotene to retinal. J. Biol. Chem. 275, 11915–11920.

Wahlberg, I., Eklund, A.-M., 1998. Degraded carotenoids. In: Britton, G., Liaaen-Jensen, S., Pfander, H. (Eds.), Carotenoids, Vol. 3: Biosynthesis and Metabolism. Birkhäuser, Basel, pp. 195–216.

Winterhalter, P., Rouseff, R. (Eds.), 2001a. Carotenoid-Derived Aroma Compounds. ACS Symp. Ser. 802, Vol. 219. American Chemical Society, Washington, DC.

Winterhalter, P., Rouseff, R., 2001b. Carotenoid-derived aroma compounds: An introduction. In: Winterhalter, P., Rouseff, R. (Eds.), Carotenoid-Derived Aroma Compounds. ACS Symp. Ser. 802, Vol. 219. American Chemical Society, Washington, DC, pp. 1–17.

Winterhalter, P., Schreier, P., 1988. Free and bound C_{13} norisoprenoids in quince (*Cydonia oblonga,* Mill.) fruit. J. Agric. Food Chem. 36, 1251–1256.

Winterhalter, P., Schreier, P., 1995. The generation of norisoprenoid volatiles in starfruit (*Averrhoa carambola* L.): a review. Food Rev. Int. 11, 237–254.

Wolf, G., 2001. The enzymatic cleavage of β-carotene: end of a controversy. Nutrition Reviews 59, 116–118.

Wyss, A., Wirtz, G., Woggon, W.D., Brugger, R., Wyss, M., Friedlein, A., Bachmann, H., Hunziker, W., 2000. Cloning and expression of β, β-carotene 15,15′-dioxygenase. Biochem. Biophys. Res. Commun. 271, 334–336.