JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Partial Purification and Kinetic Characterization of a Carotenoid Cleavage Enzyme from Quince Fruit (*Cydonia oblonga*)

PETER FLEISCHMANN,* KERSTIN STUDER, AND PETER WINTERHALTER

Institute of Food Chemistry, TU Braunschweig, Schleinitzstrasse 20,
DE 38106 Braunschweig, Germany

For the first time, a cytosolic carotenoid cleavage enzyme isolated from quince (*Cydonia oblonga*) fruit is described. The enzyme was partially purified by using centrifugation, acetone precipitation, ultrafiltration (300 kD, 50 kD), isoelectric focusing (pH 3−10), and sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5%). In this way, an enzymatically active protein fraction was obtained that contained three similar proteins, all exhibiting molecular weights in the range of 20 kD. Using β-carotene as substrate, the enzyme activity was detected spectrophotometrically at a wavelength of 505 nm. The time constant of the reaction was 8.2 min, the Michaelis constant (K_m) was 11.0 μmol·L^{-1}, and the maximum velocity (v_{max}) was 0.083 μmol·L^{-1}·min^{-1}·mg$_{(protein)}$$^{-1}$. The optimum temperature was above 50 °C.

KEYWORDS: ***β*-Carotene; fruit; oxidative cleavage; enzyme; *Cydonia oblonga***

INTRODUCTION

Volatile carotenoid breakdown products are long known as important flavor compounds in various fruits (*1*−*3*). Among others, the flavor of quince fruit (*Cydonia oblonga*) is strongly influenced by carotenoid-derived compounds (*4, 5*). However, until now the "in vivo" formation pathways of these carotenoid-related flavor compounds are not known.

Next to the abiotic oxidative breakdown of carotenoids by photochemical reactions (*6, 7*), the enzymatic oxidative degradation has to be regarded as a crucial pathway of flavor formation. But, whereas the enzymatic cleavage of carotenoids in animal tissues is well-known (*8*−*12*), there is little evidence for similar reactions in plant tissues. With the exception of enzymes specifically degrading neoxanthin to abscisic acid in a one-step reaction (*13*), there is only information about carotenoid cleavage enzymes in *Arabidopsis thaliana* (*14*) and in starfruit (*15*), both with C13 and C15 compounds as most likely reaction products.

In this paper, we describe the partial purification of the cytosolic carotenoid cleavage enzymes from quince fruit skin. The carotenases are characterized by their kinetic key parameters (v_{max}, K_m, time constant, and temperature dependence).

MATERIALS AND METHODS

Plant Materials. Quince fruit (*Cydonia oblonga*) was obtained from a local fruit market in Würzburg, Germany. To isolate an enzymatically active protein fraction, it was necessary to store the fully ripened fruit at low temperatures (4 °C) for at least 2 weeks. This period of cold storage was necessary to induce formation of an intense flavor as well as sufficient amounts of carotenoid cleavage enzymes.

* To whom correspondence should be addressed. Phone: 49-531-3917217. Fax: 49-531-3917230. E-mail: p.fleischmann@tu-bs.de.

Reagents. β-Carotene was purchased from Sigma (Steinheim, Germany). 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 Merck, Germany. All other reagents were of analytical grade.

Enzyme Purification. After removing the wax layer of fully ripened quince fruit 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) using a Teflon homogenizer (120 s, 1500 rpm). The resulting homogenate was centrifuged at 2000*g* for 10 min, and the supernatant was again centrifuged (10000*g*, 120 min). The supernatant of the second centrifugation was subjected to an acetone precipitation (85%, 2 h, 1.0 °C). The resulting precipitate was rediluted in sample buffer and subjected to a preparative isoelectric focusing (Bio-Rad Rotofor, pH 3−10). The protein fractions resulting from the isoelectric focusing were screened for carotenoid cleavage activity. To remove the ampholytes, the active fractions were ultrafiltered at 50 kD. The enzymes were stored in sample buffer. The workup was carried out at 4 °C.

Enzyme Activity. Carotenase activity was determined spectrophotometrically using β-carotene as substrate. β-Carotene (0.1 mg/mL) was dissolved in water using Tween 40 (10% in stock solution) as a detergent. The reaction was carried out in microcuvettes (final volume 375 μL) inside of the spectrophotometer, using an initial β-carotene concentration of 2.48 μmol/L in the reaction mixture. The reaction was measured directly against a β-carotene control solution which underwent the same reaction conditions, but contained no proteins. Because a dual beam spectrometer (Perkin-Elmer Lambda 7) was used, the resulting absorbance values directly reflect the enzyme-induced change in β-carotene concentration inside the reaction cuvette. Molecular oxygen, needed as second substrate for the carotenoid cleavage reaction, was provided in abundance using air-saturated sample buffer solutions. The kinetics of the carotenoid cleavage reactions were monitored continuously at 505 nm. At this wavelength, it was possible to monitor the

10.1021/jf011184j CCC: $22.00 © 2002 American Chemical Society
Published on Web 02/13/2002

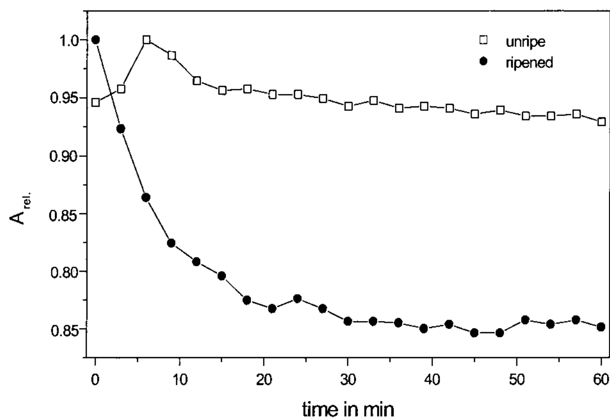

Figure 1. Kinetics of β-carotene degradation by enzymes isolated from unripe (□) and fully ripened (●) quince (*Cydonia oblonga*).

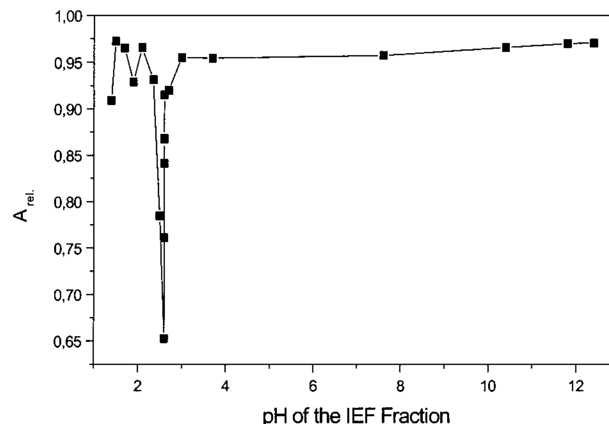

Figure 2. Activity pattern of the enzyme after preparative isoelectric focusing.

degradation of β-carotene selectively, i.e., uninfluenced from the spectral absorption of resulting cleavage products.

Temperature Dependency. The temperature-dependent enzyme activity was measured in a manner similar to the method described above. However, here only the reaction cuvettes contained β-carotene. The reference cuvettes always contained β-carotene-free, but otherwise identical, solutions. For the control measurements, these were heat deactivated (90 °C, 10 min) prior to the incubation experiments. No difference in β-carotene degradation could be found between protein-free incubation solutions and incubations containing deactivated enzymes.

Protein Determination. The protein content was measured according to the method of Bradford (*16*) using bovine serum albumin (BSA) as standard.

Electrophoresis. Denaturating polyacrylamide gel electrophoresis (SDS−PAGE) was carried out in a mini protein cell (Bio Rad) using 4.5%/7.5% discontinuous gels. The gels were stained with a commercially available silver stain kit (WAKO Corp., Japan). All runs were carried out under constant voltage conditions (30 V) at room temperature.

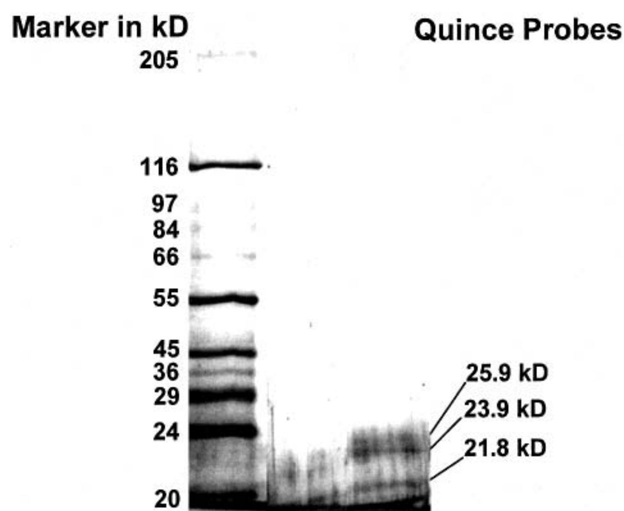

Figure 3. Polyacrylamide gel electrophoresis of the partially purified enzyme.

RESULTS AND DISCUSSION

Enzyme Purification. Despite the highly lipophilic nature of its substrate, carotenases are cytosolic, and therefore hydrophilic, proteins (*17, 18*). Consequently, they are found exclusively in water-soluble fractions of quince peelings, whereas no activity could be detected in other fractions or fruit tissues. Importantly, only extracts from fully ripened quince fruit showed carotenoid cleavage activity (**Figure 1**). After the isoelectric focusing, the enzyme activity was concentrated in a single peak (**Figure 2**). The peak fractions were pooled as partially purified carotenoid cleavage enzyme. This pooled protein fraction still contained 0.7% of the protein content of the homogenate; in other words, a 143-fold purification of the carotenoid cleavage enzymes was achieved. This is an approximate value, as our spectrometric assay did not allow a direct measurement of the enzyme activity in the still turbid protein fractions prior to the isoelectric focusing step. After SDS−PAGE and silver staining, three protein bands were detected in the enzyme solution (**Figure 3**). The molecular weight of the proteins was calculated from the r_f value of the SDS−PAGEs. The three bands at 21.8 kD, 23.9 kD, and 25.9 kD represent proteins with molecular weights clearly lower than the well-known 66 kD animal carotenases (*12, 19*). A possible explanation could be the composition of the quince enzymes out of three ∼22 kD subunits each. By comparison, the molecular weight was also determined under nondenaturating conditions (*15*). Because no differences from the results described in this paper were observed, at least

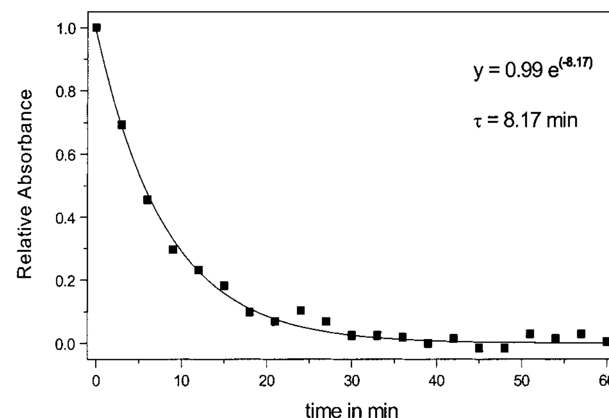

Figure 4. Time drive with regression curve and calculated time constant of the quince carotenase.

the proposed enzyme subunits should not be connected by S bonds. However, even with no evidence for smaller subunits in carotenases from other sources, the enzymes isolated from quince fruit may still consist of three subunits.

Time Constant of the Reaction. The time constant of the reaction was calculated by nonlinear regression of the β-carotene degradation time course (**Figure 4**). The value obtained was 8.2 min. **Figure 4** shows that the time course of the carotenoid

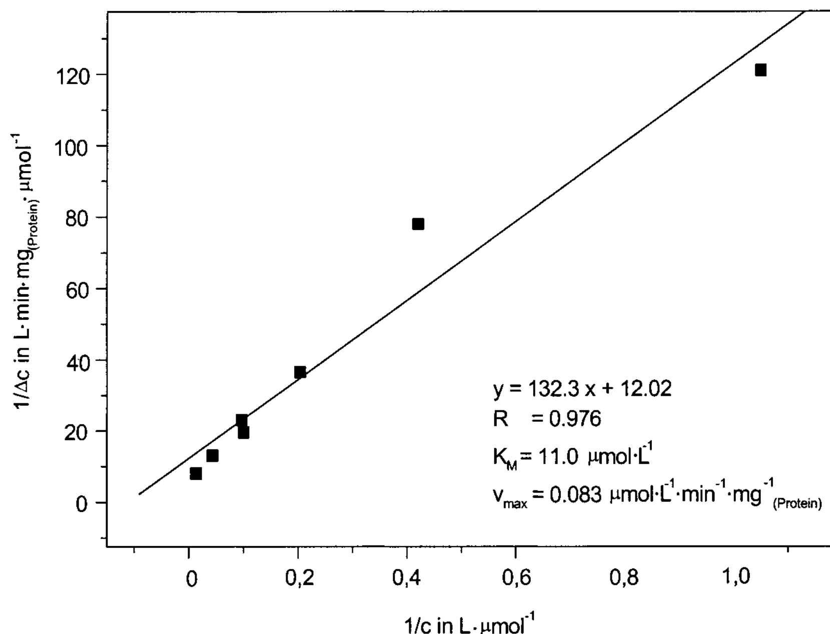

Figure 5. Lineweaver–Burke plot with regression curve and calculated kinetic key constants of the enzyme.

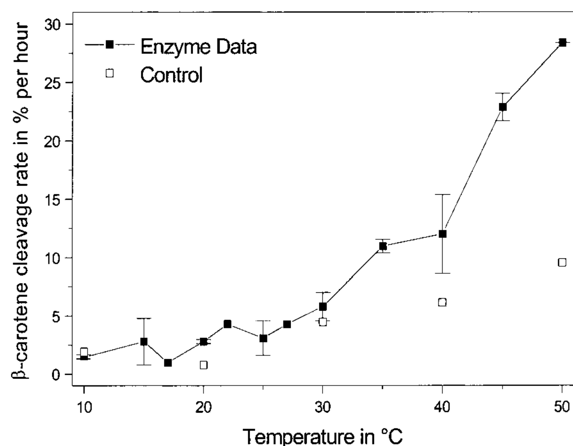

Figure 6. Effect of temperature on the enzyme. The enzyme data represent the mean ± SD of five independent experiments. The control data show typical values.

cleavage reaction could be fitted to an exponential function, typical for a first-order reaction. Under our experimental conditions, the influence of other substrates in addition to β-carotene (i.e., oxygen) is negligible. Consequently, it was possible to use the first-order Michaelis−Menten kinetics for the kinetic description of the quince carotenases.

Effect of Substrate Concentration. The kinetic parameters (v_{max} and K_m) of the carotenoid cleavage enzymes isolated from quince were studied at pH 7.0 and 23 °C. K_m and v_{max} were calculated by linear regression of the Lineweaver−Burke plot of the enzyme, using β-carotene as substrate (**Figure 5**). The values obtained ($v_{max} = 0.083\ \mu mol \cdot L^{-1} \cdot min^{-1} \cdot mg_{(protein)}{}^{-1}$; $K_m = 11.0\ \mu mol \cdot L^{-1}$) are clearly different from kinetic values determined for plant carotenases isolated from other sources (*15*). The K_m of the quince carotenases described here is approximately 4 times higher than the one reported for starfruit carotenases, whereas the v_{max} is about 10 times lower than that of starfruit (*15*). The carotenases isolated from quince skin show a clearly lower affinity to the substrate β-carotene (K_m) and also a lower turnover efficiency (v_{max}) compared with carotenases obtained from starfruit.

Effect of Temperature and pH on Enzyme Activity. The temperature profile of quince carotenases shows an increase of enzyme activity until the maximum temperature measured (**Figure 6**). Despite the increasing nonenzymatic β-carotene degradation at higher temperatures (**Figure 6**, Control), the data clearly show that the optimum temperature of these enzymes might be even higher than 50 °C. This is not unexpected, as the optimum temperature for enzyme activity of other plant carotenases was also found to be in the same range (*15*). No significant differences in β-carotene degradation activity could be found in the pH range between 6.0 and 9.0. These results differ from those reported for enzymes derived from starfruit (*15*). Considering the carotenases from quince and starfruit compared here are otherwise rather similar in their kinetic characteristics, the difference in their pH profiles is somewhat surprising. To further investigate this discrepancy, studies on the influence of the different climatic origins of the plants (quince from temperate European, starfruit from tropical) are currently planned.

CONCLUSIONS

In this paper the partial purification and kinetic characterization of carotenoid cleavage enzymes from quince (*Cydonia oblonga*) are reported. The purified fraction contained a mixture of three cytosolic proteins each of about one-third the molecular mass of the previously reported animal carotenases (∼22 vs ∼66 kDa, respectively). Despite this difference, the kinetic parameters of the quince-derived carotenases are mostly similar to other carotenases.

ACKNOWLEDGMENT

The authors thank Dr. P. Titze from Institute of Botany, and Prof. Dr. G. Seitz from Institute of Zoology, both at the University of Erlangen, Germany, for their kind support.

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Received for review September 5, 2001. Revised manuscript received December 19, 2001. Accepted December 19, 2001. This work was supported in part by a research grant from DFG. This work was presented in part at the ACS symposium "Carotenoid Derived Aroma Compounds", March 26−30, 2000, San Francisco, CA.

JF011184J