Enzymatic Hydrolysis of Carotenoid Fatty Acid Esters of Red Pepper (*Capsicum annuum* L.) by a Lipase from *Candida rugosa*

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Analyses of red pepper extracts which had been pretreated with lipase type VII (EC 3.1.1.3.) from *Candida rugosa* showed for the first time pepper carotenoid esters to be substrates of this enzyme. However, the extent of enzymatic hydrolysis depends on the respective carotenoid and was not quantitative compared to chemical saponification. After enzymatic cleavage, 67-89% of total capsanthin, 61-65% of total zeaxanthin, 70-81% of total β-cryptoxanthin and 70-86% of total violaxanthin were detected in free form. Nevertheless, the method described here offers the possibility to cleave in part several carotenoid esters originating from red pepper quickly and under comparatively mild reaction conditions. Replacement of the generally performed alkaline hydrolysis by enzymatic cleavage allows the resulting product to be used in food industry as "natural" coloring agent e.g. to colour cheese and jellies.

Introduction

Pepper fruits (*Capsicum annuum* L.) are widely used as vegetables, providing a reliable source of provitamin A. Due to their high content of various carotenes and xanthophylls, they also play a protective role in scavenging free radicals and thus may reduce the risk for the development of different cancer types (Giovannucci *et al.*, 1998; Biesalski *et al.*, 1996). This capability is assigned to the polyene chain, which is also essential for the antioxidant activity of free and esterified capsanthin and zeaxanthin (Matsufuji *et al.*, 1998). Based on the properties of these phytochemicals, fruits containing severeral carotenoids are designated in recent years as so-called "functional food". Furthermore, carotenoids act as potent coloring agents. Capsanthin, which is a unique *Capsicum* carotenoid, and violaxanthin are approved as typical food colorants, designated as E 160c and E 161e. They are used in many foods, drugs, cosmetics, and animal feed while their fields of application still increase. In red pepper, carotenoids are either present in free form or partially as well as completely esterified with fatty acids (Gregory *et al.*, 1987; Mínguez-Mosquera and Hornero-Méndez, 1994; Goda *et al.*, 1995). However, the food industry generally utilizes free carotenoids, obtained by various saponification procedures, in food preparations. Furthermore, it is known from feeding experiments in poultry industry that free carotenoids can be absorbed more easily and converted by the intestine (Nys, 2000). Another advantage of a saponification step is a concentration effect and cleanup of the crude oleoresin. For these reasons standard methods of carotenoid isolation in preparative scale and analysis methods so far require chemical saponification of the extract, causing hydrolysis of the carotenoid esters, but imply the risk of isomerization and degradation of the native carotenoids.

Only few papers have been published, which describe the use of lipase for gentle hydrolysis of carotenoid esters e.g. in human milk and serum (Khachik *et al.*, 1997; Liu *et al.*, 1998). The enzymatic cleavage of esterified pepper carotenoids, especially concerning capsanthin, the major carotenoid in red pepper fruits, has not been investigated before. The method was also applied to green pepper which contains only free carotenoids to test the influence of the enzymatic procedure on these compounds.

This study presents an investigation on lipase-catalyzed ester hydrolysis of acylated pepper carotenoids by lipase type VII of *Candida rugosa* (EC 3.1.1.3.). Under technological aspects, this procedure is interesting since the resulting product may be used as a coloring food additive, produced without the use of strong alkali.

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Experimental

Materials

Ripe red and green pepper (cultivars were not known) were obtained from local retail shops. The fruits without seeds were cut into small pieces and samples of 50 g were immediately homogenized at room temperature with an Ultra Turrax T 25 (Janke & Kunkel, Staufen, Germany) for 1 min.

Chemicals

Lutein, zeaxanthin, β-cryptoxanthin and capsanthin were generously provided by Hoffmann-La Roche (Basel, Switzerland). Violaxanthin (certified standard solution, c = 0.637 mg/l in ethanol) was purchased from VKI (Hørsholm, Denmark). For convenience, the trivial names of carotenoids are used instead of the complex IUPAC nomenclature throughout this text (Rigaudy and Klesney, 1979). Diethyl ether, light petroleum (40–60 °C), methanol, ethyl acetate, and butylated hydroxytoluene (BHT) were purchased from Merck (Darmstadt, Germany), *tert*-butylmethyl ether, ethanol, triethylamine, sodium sulfate (anhydrous), potassium dihydrogen phosphate, disodium hydrogen phosphate, calcium chloride (anhydrous), β-carotene, and β-apo-8′-carotenal from Fluka (Neu-Ulm, Germany). Bile salts (sodium cholate and sodium deoxycholate, 1:1 w/w), lipase type VII (from *Candida rugosa*), and lipase type II (crude, from porcine pancreas) were purchased from Sigma (Steinheim, Germany). The lipase activities, determined by the manufacturer, calculated from the hydrolysis rate of olive oil in one hour after 30 min incubation at 37 °C, are 700 – 1500 μmol × (min × mg)$^{-1}$ protein (*Candida rugosa*; pH 7.2) and 100 – 400 μmol × (min × mg)$^{-1}$ protein (porcine pancreas; pH 7.7), respectively. The solvents used were of analytical grade and distilled before use. For HPLC analysis, ultrapure water from a Milli-Q 185 plus apparatus (Millipore, Eschborn, Germany) was employed.

HPLC analysis

Analyses were performed with an HPLC system from Hewlett Packard, Waldbronn, Germany (autosampler, gradient pump and diode array detector (DAD) model 1050) and a column thermoregulator (Mistral, Spark, The Netherlands). A YMC C30 (250 × 4.6 mm, 5 μm) reverse phase column from YMC Europe (Schermbeck, Germany) equipped with a precolumn (Nucleosil 5 μm-C_{18}, 10 × 4.6 mm, Bischoff, Leonberg, Germany) was used and kept at 35 °C. For data acquisition and processing, an HP3D chem station software system was employed. The UV absorbance of the carotenoids was recorded at 450 nm (DAD). The injection volume was 20 μl. The mobile phase consisted of mixtures of methanol, *tert*-butylmethyl ether, water, and triethylamine (81:15:4:0.1 v/v/v/v) (A) and (6:90:4:0.1 v/v/v/v) (B). The gradient started with 10 min isocratic 100 % A, followed by a ramp to obtain 50 % B at 40 min, 100 % B at 50 min, 100 % A at 55 min and isocratic 100 % A from 55 to 60 min; flow rate, 1 ml/min.

Sample preparation

a) Extraction: Total carotenoids were extracted (three replicates, 2 g homogenized tissue each) with methanol/ethyl acetate/light petroleum (1:1:1 v/v/v) following a procedure described by Breithaupt and Schwack (2000).

b) Lipase-catalyzed ester hydrolysis: Homogenized samples (three replicates, 2 g homogenized tissue each) were vortexed for 30 s with 10 ml 0.1 M phosphate buffer (pH 7.4) in sealed glass tubes. Bile salts (30 mg) and 250 μl of a solution containing sodium chloride (3 M) and calcium chloride (75 mM) were added and the mixture was kept at 37 °C for 30 min. For enzymatic hydrolysis, 100 μl of a suspension containing 50 mg lipase/ml $CaCl_2$ solution (5 mM) were added and the mixture was incubated at 37 °C for 2 h. Extraction of the carotenoids followed the procedure described by Breithaupt and Schwack (2000).

c) Saponification: The residue resulting from the extraction procedure a) was dissolved in 50 ml diethyl ether and saponified at room temperature over night with 2.5 ml potassium hydroxide in methanol (30 g/100 ml). For complete removal of alkali the solution was washed twice with 100 ml distilled water. Saturated sodium chloride solution was added to accelerate phase separation. The organic layer was dried over anhydrous sodium sulfate, evaporated to dryness, and the residue dissolved in 2 ml methanol/*tert*-butylmethyl ether (1:1 v/v) containing 100 mg BHT/100 ml.

Standard preparation and calibration

For preparation of stock solutions, 2.5 mg lutein, zeaxanthin, β-cryptoxanthin, and β-apo-8′-carotenal were dissolved in 50 ml methanol each. Capsanthin and β-carotene were dissolved analogously in ethanol and light petroleum/ethyl acetate (1:1 v/v), respectively. Working solutions were prepared from stock solutions by diluting aliquots with methanol and were stored in brown glass tubes under nitrogen at −18 °C. All solvents used contained 100 mg BHT/100 ml. Calibration was performed in the range of 0.5 to 30 mg/l. Calibration graphs were obtained by plotting peak areas versus concentration. Limits of quantitation (LOQ) and determination (LOD) were calculated from the calibration graphs according to the recommendations of the Deutsche Forschungsgemeinschaft (1991). LOQ/LOD were determined as follows (referring to the use of 2 g sample / 2 ml final volume): capsanthin: 4.2/2.8 mg/kg, lutein: 1.2/0.8 mg/kg, zeaxanthin: 1.0/0.6 mg/kg, β-apo-8′-carotenal: 1.8/1.2 mg/kg, β-cryptoxanthin: 0.9/0.6 mg/kg, β-carotene: 0.2/0.1 mg/kg. Quantification of violaxanthin was performed using a certified standard solution (c = 0.637 mg/l in ethanol) as external standard. LOQ (0.1 mg/kg) and LOD (0.3 mg/kg) were calculated using the expression $10 \times s_0$ (s_0: standard variation of a blank) and $3 \times s_0$, respectively.

Results and Discussion

For enzymatic hydrolysis of acylated pepper carotenoids a lipase from *Candida rugosa* was employed, which has already been used for cleavage of lutein, zeaxanthin, and cryptoxanthin esters derived from human milk and serum (Khachik *et al.*, 1997; Liu *et al.*, 1998). To estimate the suitability of this enzyme for hydrolyzing typical pepper carotenoid esters, analyses of direct extracts (for determination of free carotenoids) and of chemically saponified extracts (for determination of total carotenoids), originating from the same fruit, were performed. Tables I and II give a summary of the results obtained from red and green pepper.

In the case of red pepper, only 25–30% of total capsanthin (calculated from alkaline saponification, set as 100%), 23–44% of total zeaxanthin, 46 – 58% of total β-cryptoxanthin, and 24–29% of total violaxanthin were present as free xanthophylls, depending on the fruits employed. After enzymatic cleavage, 67–89 % of total capsanthin, 61–65% of total zeaxanthin, 70–81% of total β-cryptoxanthin and 70–86% of total violaxanthin were detected in free form (Table I).

Table I. Quantification of capsanthin, zeaxanthin, β-cryptoxanthin, violaxanthin and β-carotene in direct extracts (**a**), after enzymatic cleavage with lipase (**b**), and in chemically saponified extracts (**c**) [mg/kg of fresh weight][a] in three different samples (red I–III) of red pepper.

	Extract treatment		
	a	**b**	**c**
Red I			
Capsanthin	17.4 ± 0.1	60.8 ± 1.3	68.6 ± 2.0
Zeaxanthin	1.6 ± 0.1	4.3 ± 0.1	7.1 ± 0.2
β-Cryptoxanthin	6.3 ± 0.4	7.8 ± 0.1	10.8 ± 0.1
Violaxanthin	0.9 ± 0.1	3.0 ± 0.1	3.5 ± 0.1
β-Carotene	8.8 ± 0.1	7.8 ± 0.1	7.7 ± 0.1
Red II			
Capsanthin	21.0 ± 0.2	61.6 ± 3.6	81.9 ± 2.8
Zeaxanthin	2.2 ± 0.1	5.0 ± 0.4	7.8 ± 0.4
β-Cryptoxanthin	5.3 ± 0.4	7.9 ± 0.3	9.8 ± 1.2
Violaxanthin	0.9 ± 0.1	2.4 ± 0.2	3.1 ± 0.4
β-Carotene	11.4 ± 0.4	10.8 ± 0.6	10.7 ± 1.4
Red III			
Capsanthin	26.3 ± 0.9	58.1 ± 2.3	87.1 ± 3.2
Zeaxanthin	2.4 ± 0.1	3.5 ± 0.1	5.4 ± 0.1
β-Cryptoxanthin	3.7 ± 0.1	5.6 ± 0.1	8.0 ± 0.4
Violaxanthin	0.9 ± 0.1	2.6 ± 0.1	3.7 ± 0.1
β-Carotene	8.0 ± 0.4	7.1 ± 0.1	6.8 ± 0.4

[a] Mean ± standard deviation of three independent determinations.

These results show that the percentage of the respective carotenoid, liberated by lipase, deviates from fruit to fruit, although the experimental conditions were exactly the same. In order to prove the efficiency of carotenoid extraction, homogenized samples of red pepper were spiked in additional experiments with β-apo-8′-carotenal (25 mg/g) as standard. Recoveries ($n = 3$) of β-apo-8′-carotenal were 100 ± 1.3% in direct extracts, 96 ± 0.7% after enzymatic cleavage with lipase and 94 ± 0.3% after alkaline treatment. Since the repeatability of the method was good in all cases, the fact that the percentage of substrate converted to the corresponding free carotenoid was not constant could be ascribed to the different maturity of the fruits.

The concentration of β-carotene in red pepper ranged from 8.0 to 11.4 mg/kg of fresh weight determined in direct extracts. Due to an extended

exposure to light and heat the β-carotene concentrations determined after the enzymatic cleavage work-up were lower in each case, while the enzymatic and the alkaline treatment did not differ significantly from each other (Table I). This also holds for the concentrations of free lutein (8.9 - 11.7 mg/kg) and β-carotene (2.2 - 2.3 mg/kg) in green pepper (Table II). Therefore, analytical correct results for β-carotene as well as for free lutein and violaxanthin can only be obtained by rapid analysis of direct extracts. Since the highest contents of lutein and violaxanthin in green pepper were determined in direct extracts, this method demonstrates that these carotenoids occur exclusively in their free forms in *green* pepper. Capsanthin, β-cryptoxanthin and zeaxanthin were not present in extracts of green pepper.

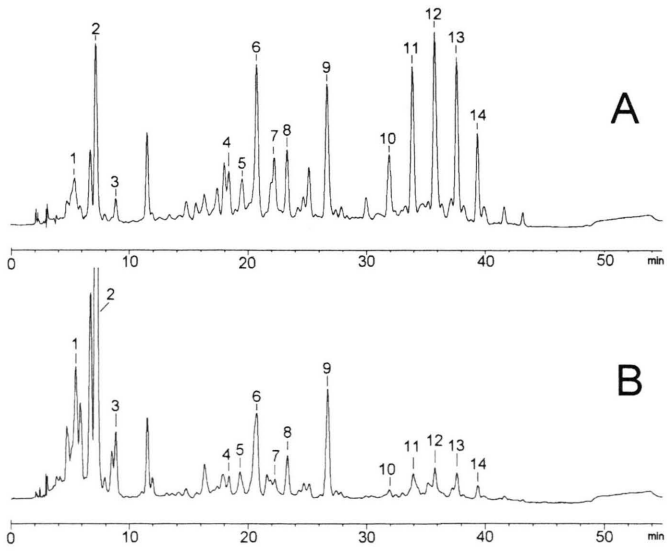
Table II. Quantification of lutein, violaxanthin and β-carotene in direct extracts (**a**), after enzymatic cleavage with lipase (**b**), and in chemically saponified extracts (**c**) [mg/kg of fresh weight][a] in three different samples (green I - III) of green pepper.

	Extract treatment		
	a	**b**	**c**
Green I			
Lutein	8.9 ± 0.5	7.7 ± 0.1	7.3 ± 0.1
Violaxanthin	1.3 ± 0.2	1.2 ± 0.1	1.1 ± 0.1
β-Carotene	2.2 ± 0.1	1.9 ± 0.1	1.8 ± 0.1
Green II			
Lutein	11.7 ± 0.1	10.7 ± 0.1	10.5 ± 0.1
Violaxanthin	2.4 ± 0.1	1.7 ± 0.2	1.3 ± 0.1
β-Carotene	2.3 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
Green III			
Lutein	9.3 ± 0.1	8.0 ± 0.3	7.6 ± 0.1
Violaxanthin	1.9 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
β-Carotene	2.2 ± 0.1	1.7 ± 0.1	1.6 ± 0.1

[a] Mean ± standard deviation of three independent determinations.

Although esterified carotenoids originating from pepper are accepted as substrates by yeast lipase, enzymatic hydrolysis does not liberate the carotenoids quantitatively. Similar observations are reported by Jacobs (1982), who used a cholesterol esterase from *Pseudomonas fluorescens* for specific hydrolysis of several carotenoid esters from crayfish and sea anemone. Figure 1 shows two typical chromatograms, obtained by HPLC analyses of a direct carotenoid extract (without further treatment) and an extract after enzymatic cleavage. The peak assignment is based on LC/MS measurements using "atmospheric pressure chemical ionisation" (APcI). Since carotenoid esters can easily be protonated (Breithaupt and Schwack, 2000), the interface was opperated in the APcI$^+$-mode, revealing singly-charged protonated molecular ions [M-H$^+$].

In the case of capsanthin, both, diesterified and monoesterified derivatives are still present after enzymatic treatment (Fig. 1, peaks 4–14). This supports the assumption, that neither diesterified



Typical HPLC chromatograms (DAD, 450 nm) of red pepper extracts: direct extract without hydrolysis (**A**) and after *Candida rugosa* lipase-catalyzed ester hydrolysis (**B**) (for conditions, see Materials and Methods). Both chromatograms are presented in the same scale. Peak assignment: **1**: violaxanthin; **2**: capsanthin (CS); **3**: zeaxanthin; **4**,**5**: 12:0-CS; **6**,**7**: 14:0-CS; **8**: 16:0-CS; **9**: β-Carotene; **10**: 12:0,12:0-CS; **11**: 12:0,14:0-CS; **12**: 14:0,14:0-CS; **13**: 14:0,16:0-CS; **14**: 16:0,16:0-CS.

nor monoesterified carotenoids are preferred substrates. Possibly a synthetic activity of the lipase has to be assumed, resulting in an equilibrium mixture of free and esterified derivatives. This effect was described by Partali (1992), who performed enzymatic esterification of optically inactive zeaxanthin with a lipase from *Candida cylindracea*.

Our investigations showed, that the concentrations of calcium and bile salts, temperature, and equilibration time do not change the degree of hydrolysis. Since the ratio of enzyme to substrate concentration may influence the reaction rate, varying concentrations of lipase were applied. However, higher concentrations of lipase did not enhance carotenoid hydrolysis. Lipase from porcine pancreas was also tested but gave generally lower yields (data not shown).

This study shows for the first time, that acylated carotenoids of red pepper may be substrates for lipase from *Candida rugosa*. If the enzyme is capable to hydrolyze carotenoid esters in general, the yields obtained here permit preparation of minor carotenoids by enzymatic hydrolysis, not available or difficult to produce at the moment. This technique is helpful especially for alkali-labile carotenoids, such as astaxanthin, fucoxanthin and actinioerythrin (Jacobs *et al.*, 1982; Aakermann *et al.*, 1996). Furthermore the method may be applied for the production of "natural" coloring agents without using alkaline treatment, suitable for use in food industry in an innovative way.

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