Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 103 (2007) 651–656
Food Chemistry
www.elsevier.com/locate/foodchem

Xanthophyll esters are hydrolysed in the presence of recombinant human pancreatic lipase

Dietmar E. Breithaupt [a,*], Alexander Alpmann [a], Frederic Carrière [b]

[a] University of Hohenheim, Institute of Food Chemistry, Garbenstraße 28, 70599 Stuttgart, Germany
[b] Laboratory of Enzymology at Interfaces and Physiology of Lipolysis, UPR 9025 CNRS, 31 Chemin Joseph Aiguier, 13402 Marseille cedex 20, France

Received 11 July 2006; received in revised form 29 August 2006; accepted 12 September 2006

Abstract

Fruit-derived bioactive xanthophyll esters have to be cleaved in the human gastrointestinal tract before absorption of free xanthophylls is possible. One candidate for ester hydrolysis is human pancreatic lipase. For estimation of their activity, an *in vitro* assay using the recombinant enzyme human pancreatic lipase (rHPL) and porcine colipase was used. Extracts of fruits were incubated with rHPL/colipase for 21 h at 37 °C. Activity of rHPL was demonstrated by an increase of free xanthophylls formed during the incubation. An extremely low activity was detected with all substrates. HPLC–(APcI)MS studies proved that lutein diesters were preferentially cleaved at the β-ionone ring. This is the first report which shows that xanthophyll esters can be cleaved in the presence of rHPL, suggesting either an unexpected secondary ester bond hydrolysis occurring within rHPL active site or, more probably, a reaction induced by other amino acids of HPL such as observed earlier with *p*-nitrophenyl acetate.

Keywords: Carotenoids; LC–MS; Recombinant human pancreatic lipase (rHPL); Xanthophyll esters

1. Introduction

Triacylglycerol digestion in humans requires pancreatic lipase (human pancreatic lipase, HPL) as a key enzyme. Since HPL is usually regarded as a 1,3-regioselective enzyme, it hydrolyzes easily primary ester groups of triacylglycerols. Secondary alcohols as 2-monoglycerides are hydrolyzed during human digestion after acyl migration of the acid from position 2 to 1 or 3 (Brockerhoff & Jensen, 1974). HPL exerts its strongest activity at the lipid–water interface of micellar or at least emulsified substrates (interfacial activation). During human digestion, generation of micelles is accomplished by various bile salts. Additional stimulation of lipolysis is caused by colipase, another enzyme synthesized by the exocrine pancreas, activating HPL in the presence of bile salts (Maylié, Charles, Gache, & Desnuelle, 1971). It is assumed that this protein allows HPL to bind to the bile salt-covered lipid–water interface. The crystal structure of the HPL–colipase complex has been published by Egloff et al. (1995).

Xanthophylls belong to the large group of secondary plant metabolites, exhibiting a wide range of biological activities. Today, it is accepted that lutein (β,ε-carotene-3,3′-diol) and zeaxanthin (β,β-carotene-3,3′-diol) protect against age-related macular degeneration (AMD) and age related cataract formation (Beatty, Boulton, Henson, Koh, & Murray, 1999; Mares-Perlman, Millen, Ficek, & Hankinson, 2002), proving both xanthophylls to be ophthalmoprotective. This was confirmed by studies of Bernstein et al. (2001), in which metabolites of zeaxanthin and lutein were isolated from human retina tissue. Since the serum concentrations of lutein and zeaxanthin are influenced by the uptake (Rock et al., 2002), supply of sufficient dietary lutein and zeaxanthin is advisible. Lutein and zeaxanthin are structurally closely related. The only difference is the position of the double bond in one ionone endring: lutein exhibits one alylic double bond, whereas zeaxanthin

* Corresponding author. Tel.: +49 711 459 4094; fax: +49 711 459 4096.
E-mail address: breithau@uni-hohenheim.de (D.E. Breithaupt).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.09.009

features two β-ionone rings with conjugated double bonds (Fig. 1). A further xanthophyll with biological activity is β-cryptoxanthin (β,β-carotene-3-ol). During its biosynthesis in plant cells, one end ring remains unsubstituted, facilitating β-cryptoxanthin to act as vitamin-A precursor. According to Cerhan, Saag, Merlino, Mikuls, and Criswell (2003), ingestion of β-cryptoxanthin together with zinc may reduce the risk of rheumatoid arthritis. Yamaguchi (2004) proposed that β-cryptoxanthin may protect against bone diseases as osteoporosis, and Yuan, Stram, Arakawa, Lee, and Yu (2003) proposed that β-cryptoxanthin acts as a chemopreventive agent for lung cancer in humans.

Especially in tropical fruits, oranges, and red peppers, xanthophylls occur esterified with various long-chain fatty acids (Breithaupt & Bamedi, 2001; Gregory, Chen, & Philip, 1987; Mínguez-Mosquera & Hornero-Méndez, 1994). During digestion, they have to be cleaved enzymatically before absorption by entherocytes takes place. Wingerath, Stahl, and Sies (1995) investigated the carotenoid pattern in chylomicrons and serum after ingestion of tangerine juice rich in β-cryptoxanthin esters. They observed increasing amounts of free β-cryptoxanthin both in the chylomicrons as well as in the serum but no β-cryptoxanthin esters were detectable. This indicates an effective ester hydrolysis prior to incorporation into lymphatic lipoproteins. Interestingly, the enzyme responsible for hydrolysis of xanthophyll esters in the human gastrointestinal tract is actually not known (Furr & Clark, 1997). It has been assumed that HPL cleaves *inter alia* fruit-derived xanthophyll esters during digestion although these substrates exhibit esters of secondary alcohols. In former studies performed in our laboratory, no activity of HPL against xanthophyll esters was found (Breithaupt, Bamedi, & Wirt, 2002a). Lipolytic HPL activity was only detectable if retinyl palmitate was used as substrate. At that time, HPL quantities commercially available were outmost limited. The successful expression of HPL in insect cells (Thirstrup et al., 1993) and yeast (Yang & Lowe, 1998) has however allowed to obtain higher amounts of the – recombinant – human enzyme (rHPL) since a few years.

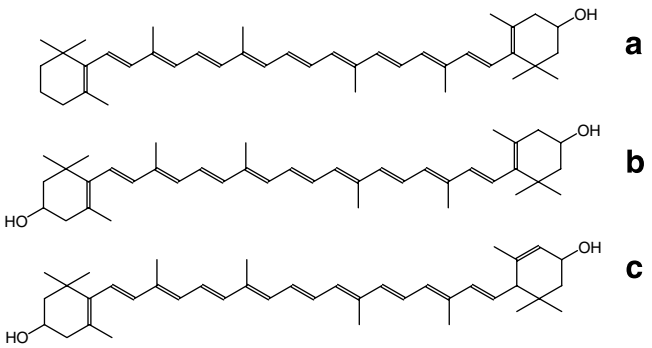

Fig. 1. Chemical structures of xanthophylls used in their acylated forms in *in vitro* rHPL-assays: (a) = β-cryptoxanthin; (b) = zeaxanthin; (c) = lutein.

For revisiting pancreatic lipase activity on xanthophyll esters, we performed an enzymatic *in vitro* assay using rHPL mixed with extracts of tangerine (*Citrus reticulata*), papaya (*Carica papaya*), wolfberries (*Lycium barbarum*) and marigold (*Tagetes erecta*) containing xanthophyll esters of β-cryptoxanthin (tangerine/papaya), zeaxanthin (wolfberries), and lutein (marigold). Since colipase was actually not available as recombinant human enzyme, porcine colipase was added to each assay.

2. Materials and methods

2.1. Chemicals and samples

Light petroleum (boiling fraction 40–60 °C), methanol, ethyl acetate and *tert*-butyl methyl ether were purchased from Merck (Darmstadt, Germany). All solvents were distilled before use. High-purity water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). Bile salts (cholic acid/deoxycholic acid sodium salt mixture 1:1) and porcine colipase (500 μg pure enzyme) were obtained from Sigma–Aldrich (Taufkirchen, Germany). Soy lecithin was a gift from Barentz GmbH, Oberhausen, Germany. β-Cryptoxanthin was generously provided by Hoffman-La-Roche (Kaiseraugst, Switzerland). Dried wolfberries (*Lycium barbarum*) were kindly provided by "Rich Nature Nutraceutical Laboraterries" WA, USA. The papaya puree (*Carica papaya*) was a gift of "Schutzgemeinschaft der Fruchtsaft-Industrie e.V. (SGF)" (Nieder-Olm, Germany) and was stored frozen at −20 °C until use. Marigold oleoresin (*Tagetes erecta*) was kindly supplied by Euram Food GmbH (Stuttgart, Germany). Direct tangerine juice (*Citrus reticulata*) was obtained from a local supermarket. Recombinant HPL (rHPL) was produced in *Pichia pastoris* using the constitutive expression system commercially available from Invitrogen GmbH (Karlsruhe, Germany) and following the experimental procedure described for the production of human pancreatic lipase-related Protein 1 (Aloulou, Grandval, De Caro, De Caro, & Carrière, 2006). The specific activity of rHPL was determined using tributyrin as substrate (pH 7.5) in presence of bile salts (0.5 mM) and porcine colipase (2:1 molar excess vs. lipase) and was found to be identical (12.500 U/mg) to that of native HPL and recombinant HPL produced in insect cells (Thirstrup et al., 1993).

2.2. Preparation of xanthophyll ester extracts

To prepare the xanthophyll ester extracts, plant samples were extracted using a ternary solvent mixture (ethyl acetate/methanol/light petroleum, 1:1:1, v/v) as follows: wolfberries (20 g) were ground using a kitchen mill. The powder was extracted using the solvent mixture until the extract was colorless. The extracts were combined (final volume: 500 mL) and the solvent evaporated in vacuum at 30 °C. To remove traces of water, ethanol (5 mL) was added

and the solvent was removed again. Finally, the residue was dissolved in a mixture of *tert*-butyl methyl ether/methanol (1:1, v/v). Papaya puree (350 g) was suspended in aliquots in the solvent mixture applying a Büchner funnel until the solid residue was colorless (final volume: 1.5 L). To assist separation of the phases, the extract was mixed with an aliquot of a saturated NaCl solution (300 mL) in a separator funnel. The organic phase was treated as described above. Tangerine juice was extracted in aliquots (150 mL) using the solvent mixture until the extracts were colorless. The resulting solution (final volume: 500 mL) was treated as described above. Marigold oleoresin (50 mg) was dissolved in *tert*-butyl methyl ether/methanol (1:1 (v/v), 100 mL) without further work-up steps. All xanthophyll ester solutions were kept at −20 °C until enzymatic assays were performed.

To determine individual xanthophyll concentrations, aliquots (2 mL) of the extracts were saponified with methanolic KOH (30 % (w/v), 5 mL) in diethyl ether (50 mL) over night. After washing the ether phase with water, the solvent was evaporated in vacuum at 30 °C, the residue dissolved in *tert*-butyl methyl ether/methanol (1:1 (v/v), 100 mL), membrane filtered (0.45 μm), and directly subjected to HPLC/DAD (450 nm) analysis.

2.3. Enzymatic in vitro assay

The assay was based on a system published previously (Breithaupt et al., 2002a). In brief: aliquots of the xanthophyll ester solutions equal to 100 nmol β-cryptoxanthin (tangerine or papaya extract), zeaxanthin (wolfberry extract) or lutein (marigold solution) were transferred in sealable glass tubes (50 mL volume). Bile salts (30 mg) and soy lecithine (18 mg) were added, and the solvent was removed in a gentle stream of nitrogen. The residue was suspended in an oil solution (2.5 g olive oil/100 mL light petroleum; 400 μL), sonicated (2 min), and the solvent removed again in a stream of nitrogen. The residue was suspended in phosphate buffer (pH 7.4, 0.1 M, 10 mL), 250 μL of a sodium chloride (3 M)/calcium chloride solution (75 mM) were added and the mixture preincubated at 37 °C for 30 min in an air oven (final volume: 10.25 mL). For enzymatic hydrolysis, suspensions containing rHPL (500 μg/500 μL) and porcine colipase (250 μg/250 μL) in phosphate buffer (pH 7.4, 0.1 M) were added. Incubation was performed at 37 °C for 21 h. Enzyme solutions were prepared daily before addition. To stop the reaction, a mixture of ethyl acetate, methanol, and light petroleum (1:1:1 (v/v), 10 mL) was added. The organic phase was removed and the extraction process repeated twice (10 mL each). The organic phases were combined, the solvent removed in vacuum at 30 °C. The residue was dissolved in *tert*-butyl methyl ether / methanol (1:1 (v/v), 2 mL), membrane filtered (0.45 μm), and subjected to HPLC/DAD (450 nm) analysis. Blank samples were constructed accordingly without the use of colipase and rHPL.

2.4. HPLC and LC/MS analyses

The HPLC system consisted of a modular system HP1100 (Hewlett-Packard GmbH, Waldbronn, Germany) with a diode array detector set to 450 nm. A YMC analytical column (YMC, Schermbeck, Germany) with 5 μm-C30-reversed phase material (250 mm × 4.6 mm i.d.) including a precolumn (10 mm × 4.0 mm i.d.) was used for separation at 35 °C. HPLC–(APcI)MS was performed on an HP1100 modular HPLC system, coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer. The MS parameters and the mobile phases have been detailed earlier (Breithaupt, Wirt, & Bamedi, 2002b). Mass spectra were acquired with MassLynx 3.2 software.

2.5. Calibration

For calibration, zeaxanthin was isolated as described earlier (Weller & Breithaupt, 2003). For quantification of β-cryptoxanthin, the reference compound (1 mg) was dissolved in light petroleum (100 mL). The concentration [μmol/L] of the β-cryptoxanthin solution was calculated photometrically based on an ε value of 131.000 [L/(mol × cm)] at 452 nm (Köst, 1988). Aliquots were evaporated, dissolved in *tert*-butyl methyl ether/methanol (1:1, v/v), and subjected to HPLC/DAD (450 nm) analysis. Calibration graphs were created by plotting calculated concentrations vs. the peak areas obtained by HPLC/DAD (450 nm) analysis. The zeaxanthin calibration graph was used for quantification of lutein, too. Xanthophyll calibration graphs were recorded in a range of 0.5–15 μmol/L.

3. Results

3.1. Xanthophyll ester pattern

Since papaya and tangerine are known for their high concentrations of β-cryptoxanthin esters (Breithaupt & Bamedi, 2001; Wingerath et al., 1995), extracts of both fruits were chosen to study rHPL activity towards xanthophyll monoesters. HPLC–(APcI)MS studies revealed the presence of different β-cryptoxanthin esters (Fig. 2, lower trace): β-cryptoxanthin laurate (C12:0; **3**) and myristate (C14:0; **4**) were the predominant components, whereas β-cryptoxanthin oleate (C18:1; **5**) and palmitate (C_{16}:0; **6**) formed minor constituents. Additionally, free β-cryptoxanthin (**1**) and β-carotene (**2**) were present. Likewise, papaya extracts showed a very typical β-cryptoxanthin ester pattern, β-cryptoxanthin laurate being the main component (Breithaupt & Bamedi, 2001; Breithaupt et al., 2002a). Thus, if papaya and tangerine extracts were studied in the *in vitro* assay, rHPL may react with β-cryptoxanthin esters with different chain lengths. Extracts of wolfberries were clearly dominated by zeaxanthin dipalmitate (**8**, Fig. 3, lower trace), other xanthophylls were present in outmost low extents (Li, Peng, & Zhang, 1998; Weller & Bre-

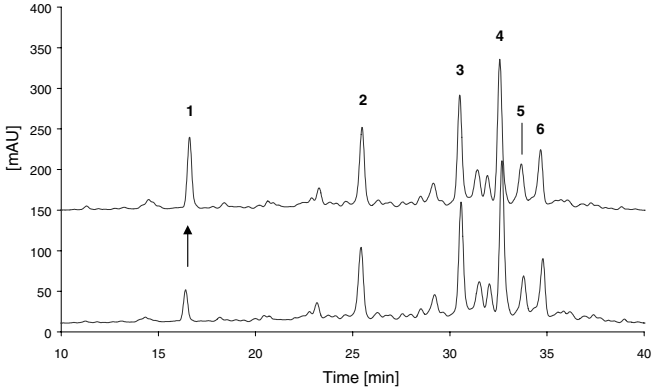

Fig. 2. Section of a typical HPLC chromatogram (DAD, 450 nm) of a native tangerine extract (*Citrus reticulata*) before (lower trace) and after (upper trace) incubation with rHPL/colipase (21 h, 37 °C). Peak assignment (Cr = β-cryptoxanthin): (**1**) = freer Cr, (**2**) = β-carotene, (**3**) = Cr-laurate, (**4**) = Cr-myristate, (**5**) = Cr-oleate, (**6**) = Cr-palmitate.

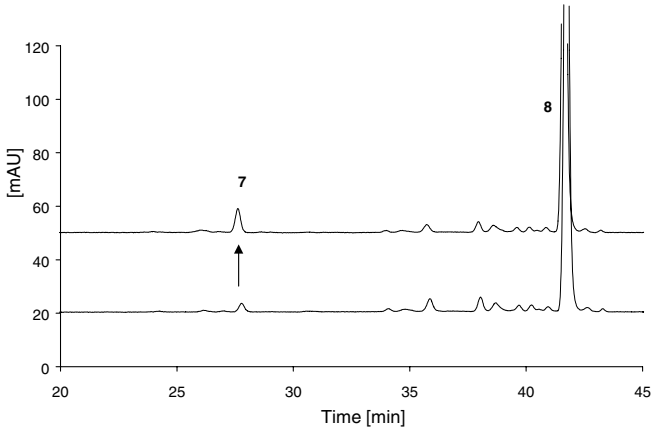

Fig. 3. Section of a typical HPLC chromatogram (DAD, 450 nm) of a native wolfberry extract (*Lycium barbarum*) before (lower trace) and after (upper trace) incubation with rHPL/colipase (21 h, 37 °C). Peak assignment (Z = zeaxanthin): (**7**) = Z-monopalmitate, (**8**) = Z-dipalmitate.

ithaupt, 2003). This unique situation provides the opportunity to study the enzymatic activity with only one component. Marigold extracts typically comprise various lutein diesters: mixed as well as homogenous esters with saturated acyl chains (C_{12}, C_{14}, C_{16}, C_{18}) formed principle components (Fig. 4, lower trace) (Breithaupt et al., 2002b; Hadden et al., 1999).

3.2. Enzymatic reactions

To ensure maximum reactivity in the *in vitro* system, porcine colipase (10 kDa) was added to the enzymatic assays on a 2:1 molar basis relative to rHPL (50 kDa) (Bezzine et al., 1999). Colipase is actually not available as recombinant human protein, forestalling simulation of an *in vitro* assay mimicking completely human digestion. However, it is known that porcine colipase will act in a similar way as the human enzyme does. Bile salts, which are essential for micelle formation and solubilization of lipolysis products, were present in the *in vitro* assay in a concentration of 30 mg/10.25 mL (total assay volume). Taking into account the molecular mass of cholic acid (408.6 g/mol), this corresponds to a concentration of 7.2 mM which is roughly equal to that present in the human digestion tract (8–12 mM) after ingestion of a meal (Hernell, Staggers, & Carey, 1990). Soy lecithin and olive oil were added to assist formation of mixed micelles and lipid droplets. Enzymatic reactions were carried out in an air oven at 37 °C. Thus, the *in vitro* assay applied resembles quasi-physiological conditions found in the human digestion tract with respect to hydrolysis of xanthophyll esters. It is understood that the reaction time of 21 h is relatively long with respect to conditions found during human digestion. However, as only small amounts of xanthophylls were formed during enzymatic hydrolysis, such a long time was needed to allow for reliable calculation of the enzymatic activity of rHPL.

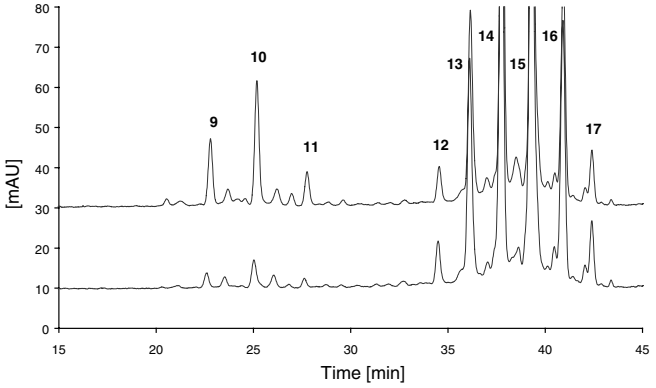

Fig. 4. Section of a typical HPLC chromatogram (DAD, 450 nm) of a native marigold extract (*Tagetes erecta*) before (lower trace) and after (upper trace) incubation with rHPL/colipase (21 h, 37 °C). Peak assignment (L = lutein): (**9- 11**) = L–3′O-acyl derivatives, (**12**) = L-laurate/myristate, (**13**) = L-dimyristate, (**14**) = L-myristate/palmitate, (**15**) = L-dipalmitate, (**16**) = L-palmitate/stearate, (**17**) = L-distearate.

Investigating tangerine and papaya extracts, rHPL hydrolyzed various β-cryptoxanthin esters, resulting in an increase of the respective β-cryptoxanthin peak (Fig. 2, upper trace). However, conversion was not complete after 21 h, suggesting that the reaction rate was much slower than those usually measured with typical rHPL substrates such as tributyrin (12.500 μmol FFA released per min under optimised conditions). Zeaxanthin dipalmitate was hydrolyzed to a small extent, resulting in a moderate increase of the peak area corresponding to zeaxanthin monopalmitate (Fig. 3). However, rHPL activity was definitely proven by comparison with chromatograms obtained for blank samples (without addition of rHPL/colipase). Remarkably, the free xanthophyll was not detected after a reaction time of 21 h. The same phenomenon occurred when lutein diesters from marigold were used as rHPL substrates: different monoesters were formed, but the free xanthophyll was not present in the reaction mixtures. HPLC–(APcI)MS analyses of lutein assay extracts revealed unexpected results: the main peaks corresponding to lutein monoesters (**9**–**11**, Fig. 4, upper trace) showed an

intense fragment ion at m/z 551.4, proving that the respective lutein monoesters were still acylated at the ε-ionone ring. This assignment is in agreement with lutein diester fragmentation studies published previously by our group (Breithaupt et al., 2002b). Consequently, rHPL preferentially hydrolyzed ester bonds of the lutein β-ionone ring. Thus, there is an interesting selectivity of rHPL towards acyl chains bound to different ionone ring structures.

3.3. Calculation of rHPL activity

The specific activity of rHPL towards xanthophyll esters was estimated from the amount of xanthophyll liberated per min [1 U/mg rHPL = μmol/(min × mg)]. Since papaya and tangerine extracts already contained small amounts of free β-cryptoxanthin, the respective concentrations were determined in additional analyses and subtracted from those generated by enzymatic ester hydrolysis. The following specific activities were calculated: papaya and tangerine (conversion of β-cryptoxanthin esters to free β-cryptoxanthin): 3.8×10^{-5} U/mg and 1.9×10^{-5} U/mg, respectively; wolfberries (conversion of zeaxanthin dipalmitate to zeaxanthin monopalmitate): 5.8×10^{-6} U/mg; marigold (conversion of lutein diesters to lutein monoesters): 1.6×10^{-5} U/mg. Thus, these specific activities were extremely low as compared to those measured with triacylglycerides as substrate. The highest of these activities was against β-cryptoxanthin esters from papaya and lowest against zeaxanthin dipalmitate from wolfberries as substrates. Activities calculated earlier for porcine pancreatic lipase and β-cryptoxanthin esters from papaya and loquat (*Eriobotrya japonica*) were in the same range (5.8×10^{-6} U/mg) for both substrates (Breithaupt et al., 2002a) and assist the data calculated for the recombinant human enzyme. Since no new xanthophyll esters appeared in the respective chromatograms, rHPL did not show transesterification activity.

Because papaya and tangerine extracts were composed of various β-cryptoxanthin esters, it was possible to estimate rHPL activity towards substrates with different acyl chain lengths and identical backbone. The β-cryptoxanthin ester pattern of tangerine is concise. Thus, corresponding assays with and without rHPL addition were chosen for detailed analyses. Comparison of the chromatograms revealed the following changes of individual β-cryptoxanthin ester peak areas (−5% C12:0; −5% C14:0; −9% C16:0; +2% C18:1). This points to a however slight preference of β-cryptoxanthin palmitate as substrate.

4. Discussion

Many esters were tested as pancreatic lipase substrates in the past and only the ester bonds with primary alcohols were found to be hydrolyzed whereas the esters with secondary alcohols were not (Derbesy & Naudet, 1972). This holds also for food-derived triacylglycerides as substrates. HPL is therefore typically described as an 1,3-regioselective enzyme. The results of this study document unambiguously that rHPL accepts native esters of xanthophylls (β-cryptoxanthin, lutein, zeaxanthin) as substrates although xanthophylls are secondary alcohols. Thus, these results can be considered as the first evidence of secondary ester bond hydrolysis by HPL.

The conversion rates were however found to be extremely low. It might be possible that the secondary ester bond of triacylglycerides is also cleaved by HPL, but at such a low rate only hydrolysis of ester bonds at position sn-1 and sn-3 can be observed under common assay conditions. Another explanation might be that the cleavage reaction observed in the present study may result from side activities of reactive residues on the surface of the protein or by special parts of the enzyme, e.g. the C-terminal domain, and not by the catalytic domain of rHPL. It has been previously shown that the C-terminal domain of pancreatic lipase exhibits the same low activity against *p*-nitrophenyl acetate (which is an ester of a secondary alcohol, too) as the entire lipase (De Caro, Chautan, Rouimi, & Rovery, 1988). Consequently, exceptional care has been taken to perform blank samples without addition of rHPL/colipase. Because no activity was found in those assays, it was concluded that the activity determined was really due to rHPL.

It is understood that the low enzymatic activity of rHPL can hardly explain the high amounts of free xanthophylls found in human blood after ingestion of xanthophyll diester-containing diets (Bowen, Herbst-Espinosa, Hussain, & Stacewicz-Sapuntzakis, 2002). Thus, the results suggest that HPL is not the key enzyme responsible for xanthophyll ester cleavage in the human gastrointestinal tract. Previous experiments with porcine cholesterol esterase (ChE) showed that this enzyme is a likely candidate (Breithaupt et al., 2002a). If human ChE will be available in future as recombinant enzyme, the *in vitro* assay described may be used to confirm this assumption and to calculate specific activities towards native fruit-derived xanthophyll esters.

Acknowledgement

The authors would like to express their gratitude to Ms. Isabelle Douchet for purification of the rHPL used in this study at the ''Laboratory of Enzymology at Interfaces and Physiology of Lipolysis'', Marseille, France. Special thanks goes to Prof. Dr. Wolfgang Schwack, University of Hohenheim, for the excellent working conditions at the Institute of Food Chemistry.

References

Aloulou, A., Grandval, P., De Caro, J., De Caro, A., & Carrière, F. (2006). Constitutive expression of human pancreatic lipase-related protein 1 in *Pichia pastoris*. *Protein Expression and Purification, 47*, 415–421.

Beatty, S., Boulton, M., Henson, D., Koh, H.-H., & Murray, I. J. (1999). Macular pigment and age-related macular degeneration. *British Journal of Ophthalmology, 83*, 867–877.

Bernstein, P. S., Khachik, F., Carvalho, L. S., Muir, G. J., Zhao, D.-Y., & Katz, N. B. (2001). Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. *Experimental Eye Research, 72*, 215–223.

Bezzine, S., Ferrato, F., Ivanova, M. G., Lopez, V., Verger, R., & Carrière, F. (1999). Human pancreatic lipase: Colipase-dependence and interfacial binding of lid domain mutants. *Biochemistry, 38*, 5499–5510.

Bowen, P. E., Herbst-Espinosa, S. M., Hussain, E. A., & Stacewicz-Sapuntzakis, M. (2002). Esterification does not impair lutein bioavailability in humans. *Journal of Nutrition, 132*, 3668–3673.

Breithaupt, D. E., & Bamedi, A. (2001). Carotenoid esters in vegetables and fruits: A screening with emphasis on β-cryptoxanthin esters. *Journal of Agricultural Food Chemistry, 49*, 2064–2070.

Breithaupt, D. E., Bamedi, A., & Wirt, U. (2002a). Carotenol fatty acid esters: Easy substrates for digestive enzymes? *Comparative Biochemistry and Physiology B, 132*, 721–728.

Breithaupt, D. E., Wirt, U., & Bamedi, A. (2002b). Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography–mass spectrometry. *Journal of Agricultural Food Chemistry, 50*, 66–70.

Brockerhoff, H., & Jensen, R. G. (1974). *Lipolytic enzymes*. New York, San Francisco, London: Academic press.

Cerhan, J. R., Saag, K. S., Merlino, L. A., Mikuls, T. R., & Criswell, L. A. (2003). Antioxidant micronutrients and risk of rheumatoid arthritis in a cohort of older women. *American Journal of Epidemiology, 157*, 345–354.

De Caro, J. D., Chautan, M. P., Rouimi, P., & Rovery, M. (1988). Acetylation of Lys-373 in porcine pancreatic lipase after reaction of the enzyme or its C-terminal fragment with *p*-nitrophenyl acetate. *Biochimie, 70*, 1785–1790.

Derbesy, M., & Naudet, M. (1972). Influence of the structure of the alcohol on the reactivity of esters of fatty acids in comparison with pancreatic lipase. *Revue Française des Corps Gras, 19*, 225–232.

Egloff, M.-P., Marguet, F., Buono, G., Verger, R., Cambillau, C., & van Tilbeurgh, H. (1995). The 2.46 Å resolution structure of the pancreatic lipase–colipase complex inhibited by a C_{11} alkyl phosphonate. *Biochemistry, 34*, 2751–2762.

Furr, H. C., & Clark, R. M. (1997). Intestinal absorption and tissue distribution of carotenoids. *Nutritional Biochemistry, 8*, 364–377.

Gregory, G. K., Chen, T.-S., & Philip, T. (1987). Quantitative analysis of carotenoids and carotenoid esters in fruits by HPLC: Red bell peppers. *Journal of Food Science, 52*, 1071–1073.

Hadden, W. L., Watkins, R. H., Levy, L. W., Regalado, E., Rivadeneira, D. M., van Breemen, R. B., et al. (1999). Carotenoid composition of marigold (*Tagetes erecta*) flower extract used as nutritional supplement. *Journal of Agricultural Food Chemistry, 47*, 4189–4194.

Hernell, O., Staggers, J. E., & Carey, M. C. (1990). Physical–chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 2. Phase analysis and aggregation states of luminal lipids during duodenal fat digestion in healthy adult human beings. *Biochemistry, 29*, 2041–2056.

Köst, H.-P. (1988). *CRC handbook of chromatography plant pigments* (Vol. 1). Boca Raton, FL: CRC Press.

Li, Z., Peng, G., & Zhang, S. (1998). Separation and determination of carotenoids in Fructus lycii by isocratic non-aqueous reversed-phase liquid chromatography. *Chinese Journal of Chromatography, 16*, 340–343.

Mares-Perlman, J. A., Millen, A. E., Ficek, T. L., & Hankinson, S. E. (2002). The body of evidence to support a protective role for lutein and zeaxanthin in delaying chronic disease – Overview. *Journal of Nutrition, 132*, 518S–524S.

Maylié, M., Charles, M., Gache, C., & Desnuelle, P. (1971). Isolation and partial identification of a pancreatic colipase. *Biochemica Biophysica Acta, 229*, 286–289.

Mínguez-Mosquera, M. I., & Hornero-Méndez, D. (1994). Changes in carotenoid esterification during the fruit ripening of *Capsicum annum Cv. Bola*. *Journal of Agricultural Food Chemistry, 42*, 640–644.

Rock, C. L., Thornquist, M. D., Neuhouser, M. L., Kristal, A. R., Neumark-Sztainer, D., Cooper, D. A., et al. (2002). Diet and lifestyle correlates of lutein in the blood and diet. *Journal of Nutrition, 132*, 525S–530S.

Thirstrup, K., Carrière, F., Hjorth, S., Rasmussen, P. B., Wöldike, H., Nielsen, P. F., et al. (1993). One-step purification and characterization of human pancreatic lipase expressed in insect cells. *FEBS Letters, 327*, 79–84.

Weller, P., & Breithaupt, D. E. (2003). Identification and quantification of zeaxanthin esters in plants using liquid chromatography–mass spectrometry. *Journal Agricultural Food Chemistry, 51*, 7044–7049.

Wingerath, T., Stahl, W., & Sies, H. (1995). β-Cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. *Archives of Biochemistry and Biophysics, 324*, 385–390.

Yamaguchi, M. (2004). Osteogenesis promoter containing β-cryptoxanthin as the active ingredient. PCT Int. Appl., WO 2004037236.

Yang, Y., & Lowe, M. E. (1998). Human pancreatic triglyceride lipase expressed in yeast cells: Purification and characterization. *Protein Expression and Purification, 13*, 36–40.

Yuan, J.-M., Stram, D. O., Arakawa, K., Lee, H.-P., & Yu, M. C. (2003). Dietary cryptoxanthin and reduced risk of lung cancer: The Singapore Chinese health study. *Cancer Epidemiology Biomarkers and Prevention, 12*, 890–898.