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A Peroxidase from *Lepista irina* Cleaves β,β-Carotene to Flavor Compounds

Holger Zorn[1,*], Sabine Langhoff[1], Manuela Scheibner[1], Manfred Nimtz[2] and Ralf G. Berger[1]

[1] Zentrum Angewandte Chemie, Institut für Lebensmittelchemie der Universität Hannover, Wunstorfer Straße 14, D-30453 Hannover, Germany
[2] Department of Structural Biology, GBF – German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany

*Corresponding author

Extracellular liquid of the edible fungus *Lepista irina* was found to effectively degrade β,β-carotene. β-Ionone, β-cyclocitral, dihydroactinidiolide, and 2-hydroxy-2,6,6-trimethylcyclohexanone were formed as volatile breakdown products of β,β-carotene with mycelium-free culture supernatants, whereas β-apo-10'-carotenal was identified as non-volatile degradation product. The key enzyme catalyzing the oxidative cleavage of β,β-carotene was purified with an overall yield of 63% and a purification factor of 43. Biochemical characterization showed a molecular mass of 50.5 kDa and an isoelectric point of 3.75. Fastest β,β-carotene degradation occurred at 34 °C and pH values between 3.5 and 4. Degenerate oligonucleotides were derived from N-terminal and internal amino acid sequences. By means of PCR-based cDNA-library screening a 1284 bp cDNA was identified which showed great overall similarity to *Pleurotus eryngii* polyvalent peroxidases. The obtained sequence contains an open reading frame of 1083 nucleotides, encoding a polypeptide of 361 amino acids. A 30 amino acid signal peptide was identified upstream of the N-terminal sequence of the mature enzyme. The *L. irina* versatile peroxidase represents the first microbial enzyme capable of carotenoid degradation that has been characterized on a molecular level, proving the participation of extracellular enzymes of white rot fungi in biotic carotenoid degradation processes.

Key words: Basidiomycete / cDNA / Cleavage / Degradation / Norisoprenoids

Introduction

Although some 100 million tons of carotenoids are biosynthesized and subsequently degraded naturally every year (Britton *et al.*, 1999), the knowledge of environmental carotenoid degradation is still fragmentary. Abiotic factors, such as heat or light, are well understood (*e.g.* Handelman *et al.*, 1991; Beatriz *et al.*, 1993), however, surprisingly few data have become available on the biotic carotenoid degradation by microorganisms. Lutein from marigold flowers (*Tagetes erecta*) was degraded by mixed cultures of *Geotrichum* sp. and *Bacillus* sp. (Sanchez-Contreras *et al.*, 2000) and a cellular, membrane-bound unstable carotene dioxygenase from the cyanobacterium *Microcystis* cleaved β,β-carotene into two molecules, β-cyclocitral (C10) and crocetindial (C20) (Jüttner *et al.*, 1985). In a recent study, more than 50 filamentous fungi and yeasts, known for *de novo* synthesis or biotransformation of mono-, sesqui-, tri-, or tetraterpenes, were screened for their capability to degrade β,β-carotene. With the basidiomycetous fungus *Marasmius scorodonius* effective cleavage of β,β-carotene was observed, and the responsible enzyme was partially characterized (Zorn *et al.*, 2003).

There is an enormous interest of the detergent, food and perfume industry in carotenoid breakdown products, as they often represent highly potent aroma compounds (Winterhalter and Rouseff, 2002). Among the cleavage products, the so-called norisoprenoids, the C13-cleavage products are of particular interest. Prominent examples are the flowery smelling β-ionone and β-damascenone, featuring threshold values of 0.007 and 0.009 μg l^{-1} (in water), respectively. As the occurrence of norisoprenoids in natural sources is restricted to trace amounts and their extraction turned out to be laborious and costly, biotechnological processes have been aspired. The co-oxidation of carotenoids, using mainly lipoxygenase or xanthine oxidase systems, was intensely investigated. Thereby carotenoids are oxidized by free radical species generated from another substrate by enzymatic reactions (*e.g.* Wu and Robinson, 1999; Wache *et al.*, 2002). Here we report on the direct cleavage of β,β-carotene to flavor compounds by a novel *L. irina* versatile peroxidase, proving the participation of extracellular enzymes of white rot fungi in natural carotenoid degradation processes.

Results

When the edible fungus *L. irina* was cultivated on a β,β-carotene-containing growth agar, a pronounced bleaching zone was formed around the mycelium after a few days. Assuming that secreted enzymes are responsible for the apparent carotenoid degradation, bioconversion of β,β-carotene was attempted using extracellular liquid of *L. irina*. For this purpose, *L. irina* was grown in submerged cultures and culture supernatant was gathered

after 10 culture days. To ensure smooth bioavailability, β,β-carotene was emulsified with Tween 40 prior to the addition to the extracellular liquid. After an incubation period of 14 h, nearly complete degradation (≥99%) of β,β-carotene was observed. β-Ionone was the main volatile metabolite (up to 12 mol%), whereas β-cyclocitral (2 mol%), dihydroactinidiolide (4 mol%), and 2-hydroxy-2,6,6-trimethylcyclohexanone (6 mol%) were formed in minor quantities (Figure 1). An HPLC method was developed to identify non-volatile degradation products of β,β-carotene. Trace amounts of β-apo-10'-carotenal (MH^+=377.35) were detected by HPLC-MS in the $APcI^+$ mode (Figure 2). Degradation of β,β-carotene with the blank tests (using buffer solution instead of culture supernatant) was <8% under these conditions, and no cleavage products of β,β-carotene were detectable. No *de novo* biosynthesis of terpenoid compounds was observed in submerged cultures and culture supernatants of *L. irina* without addition of β,β-carotene.

Kinetic investigations of the β,β-carotene degradation by mycelium-free culture media of *L. irina* indicated a rapid reaction profile. More than 90% of the initially added β,β-carotene were degraded within 30 minutes. The maximum concentration of the main product β-ionone was reached after 1 h (Figure 3).

Prior to the purification of the enzyme, its isoelectric point was determined by use of an 'activity bleaching' test. Crude enzyme concentrates were analyzed by IEF gel electrophoresis. Covering the focussed gel with a β,β-carotene containing agar layer gave a distinct bleaching band. By comparing the position of the bleaching band to Coomassie Blue-stained reference proteins, the pI of the β,β-carotene degrading enzyme was found to be 3.75.

Further biochemical properties of the β,β-carotene de-

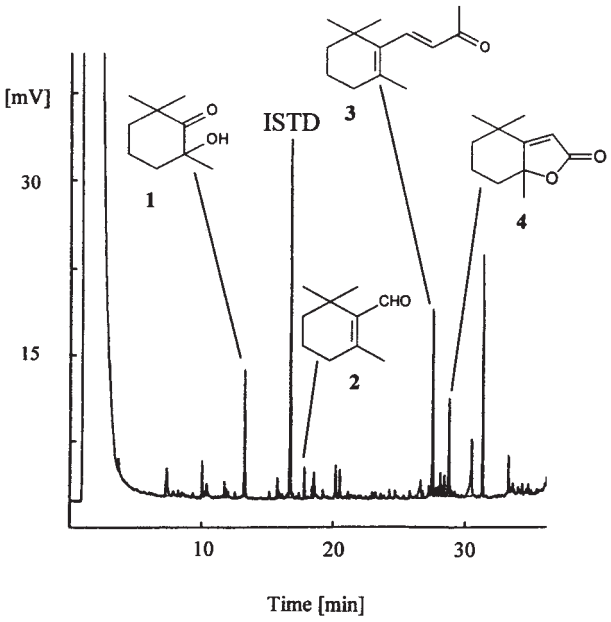

Fig. 1 Cleavage of β,β-Carotene to Volatile Compounds by Mycelium-Free Culture Supernatant of *L. irina* (GC/FID Chromatogram).
1: 2-Hydroxy-2,6,6-trimethyl-cyclohexanone; **2**: β-cyclocitral; **3**: β-ionone, **4**: dihydroactinidiolide; ISTD: α-terpineol.

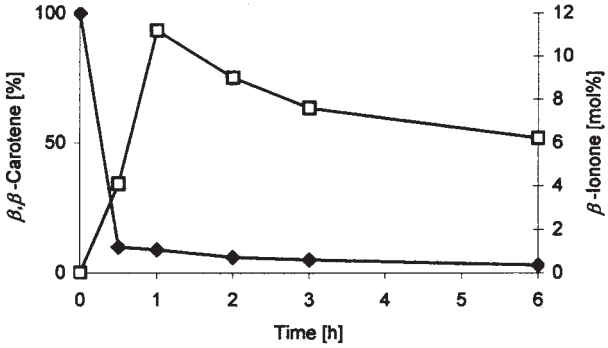

Fig. 3 Time Dependence of β,β-Carotene Degradation (◆) and Formation of β-Ionone (□) with Mycelium-Free Culture Supernatant of *L. irina*.

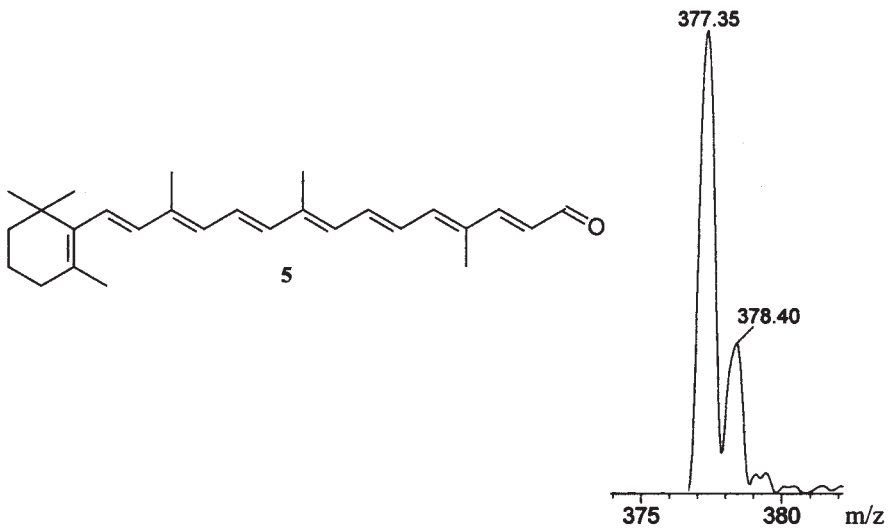

Fig. 2 Quasi-Molecular Ion (MH^+) Determined by LC/MS and Structure of β-Apo-10'-Carotenal (**5**).

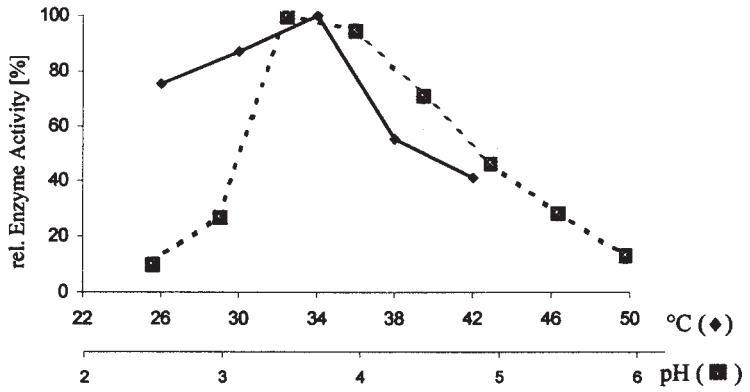

Fig. 4 Temperature Optimum and pH-Dependence of *L. irina* Versatile Peroxidase Activity.

grading *L. irina* enzyme were determined by use of a fast and efficient enzyme assay. Best β,β-carotene degradation occurred at 34 °C and the pH optimum was observed between 3.5 and 4 (Figure 4).

A purification method was developed to isolate the β,β-carotene degrading enzyme from extracellular liquid of *L. irina*. Culture supernatant was harvested after 10 culture days, when the specific activity in the medium was maximal (Figure 5). The maximum of specific activity coincided with the complete consumption of the added glucose (data not shown), indicating the fungus' need to utilize new carbon sources. Ultrafiltration as the first step

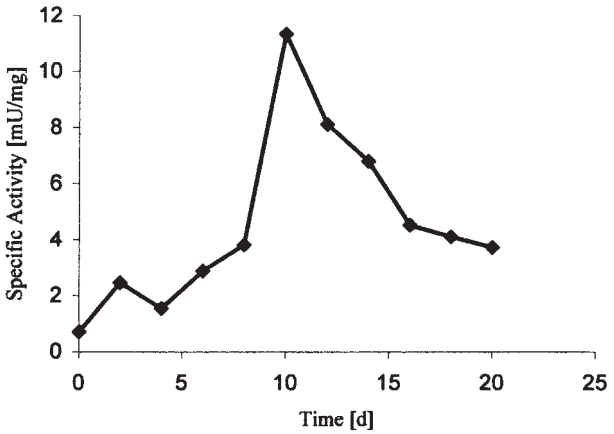

Fig. 5 Specific Activity of *L. irina* Versatile Peroxidase in the Culture Supernatant over the Cultivation Period.

was followed by three chromatographic separation procedures, including hydrophobic interaction, anion exchange, and size exclusion. An overall yield of 63% and a purification factor of 43 were achieved (Table 1). A single peak was obtained in the final SEC, and the native molecular mass was calculated to be 50 kDa from a calibration curve. The UV-VIS absorption spectrum of the purified enzyme was characteristic for heme enzymes, exhibiting an absorption maximum of 408 nm. Both, denaturing SDS-PAGE and native IEF electrophoresis of the purified protein sample showed one main band (mol. mass 50.5 kDa; pI=3.75) and a very faint second protein band (mol. mass 45 kDa, pI=3.5) close by. Further separation of these two proteins was not achieved by chromatographic methods.

Edman sequencing resulted in identical N-termini for both proteins: H_2N-AT(C)ADGRTTANAA(C)(C)VLFPILDDIQENLFDGAQ(C)CGE(E), where amino acids in parenthesis are tentative calls. Additional sequence information of the main band was obtained from ESI-MS-MS analyses: (L/I)FPGTAD...QSPLQGE(L/I)R. A cDNA library of *L. irina* was constructed, and the library was screened by polymerase chain reaction (PCR) using primer sets designed according to the vector sequence of λTriplEx2 multiple cloning site and according to the translated degenerate sequence of the internal peptide. By means of primer walking, a 1284 bp cDNA was sequenced that showed great overall similarity [98% using the WU-Blast2 (Gish, 1996–2003) algorithm] to *Pleurotus eryngii* polyvalent peroxidase MnPL2 (AF007222) and

Table 1 Purification of Extracellular β,β-Carotene Cleavage Enzyme from *Lepista irina*, Starting from 220 ml of Mycelium-Free Culture Supernatant (10th Culture Day).

Step	Total protein (mg)	Activity (mU)	Specific activity (mU mg^{-1})	Yield (%)	Purification factor
Medium	11.4	75.7	6.6	100	
Ultrafiltrate	6.0	63.6	10.6	84	1.6
HIC	0.9	55.3	64.6	73	9.8
IEC	0.5	52.6	112.4	69	17.0
SEC	0.17	47.3	281.5	63	42.7

MnPL1 (AF007221) precursors in the EMBL FASTA data base (Pearson and Lipman, 1988). The obtained sequence contains an open reading frame (ORF) of 1083 nucleotides, encoding a polypeptide of 361 amino acids. A 30 aa signal peptide was identified before the N-terminal sequence of the mature enzyme, after a KR sequence reported to be involved in protease processing (Ruiz-Duenas *et al.*, 1999). The molecular mass of the processed protein was calculated as 34.6 kDa. Using the purified enzyme, the biotransformation of β,β-carotene resulted in comparable results to those with crude culture supernatant.

Discussion

The scent of mature fruiting bodies of *L. irina* has been described as sweet, flowery, and violet-like (Gerhardt, 1997). This characteristic has become manifest *e.g*. in the Latin species name '*irina*' (=iris) or in the German trivial name 'Veilchen-(=violet)-Rötelritterling'. However, no odorous volatiles were detected when *L. irina* was grown in submerged cultures in the absence of β,β-carotene. Whereas no fungal *de novo* biosynthesis was observed under the conditions used, norisoprenoidic flavor compounds were formed upon addition of β,β-carotene to culture supernatants, resulting from highly efficient degradation of β,β-carotene. *L. irina* is a well-known and valued edible fungus, and thus represents a non-toxic microorganism for potential biotechnological applications. The spectrum of β,β-carotene cleavage products obtained with mycelium free culture supernatants (*cf*. Figure 1) resembles substantially that of co-oxidation approaches using either lipoxygenase or xanthine oxidase as biocatalysts (*e.g*. Grosch *et al.*, 1976; Wu and Robinson, 1999). The formation of the C13 ketone β-ionone as main volatile product as well as the detection of the complementary C27 aldehyde β-apo-10'-carotenal indicates an initial oxidative cleavage of the C9-C10 double bond of the β,β-carotene skeleton. Recent molecular mechanics calculations revealed the C9-C10 double bond in (*Z*)-and (*E*)-conformation to be the thermodynamically preferred point of attack for oxidative cleavage of β,β-carotene (Mohamed *et al.*, 2001). Compared to co-oxidation experiments using the xanthine oxidase/acetaldehyde system (molar yields of β-ionone of ~1%; Bosser and Belin, 1994), significantly higher molar yields of β-ionone (13 mol%) were obtained with the extracellular liquid of *L. irina*. The overall molar yields of volatile metabolites added up to maximal 25%, and only traces of the non-volatile β,β-carotene degradation product β-apo-10'-carotenal were detectable in the transformation medium. The reason for this balance gap is not yet clear. Evaporation losses of highly volatile compounds may be taken into consideration as well as further degradation of primarily formed products to readily water-soluble substances. The transformation of β-ionone has been described for several microorganisms. Complex mixtures of metabolites, comprising oxygenated and side chain degradation products, were obtained (Winterhalter, 1996; Grivel *et al.*, 1999; Grivel and Larroche, 2001).

By means of ultrafiltration and fast protein liquid chromatography, the β,β-carotene cleaving enzyme was enriched 43-fold from culture supernatant. A single protein peak was obtained in the final size exclusion chromatography; SDS and IEF gel electrophoresis both showed the presence of an 'impurity' that could not be separated by chromatographic methods. As MALDI mass fingerprints of both bands proved to be virtually identical and N-terminal amino acid sequencing gave equal N-termini for the spots, the second protein possibly represents an isoenzyme encoded by a different gene (as reported *e.g*. for peroxidases from *Phanerochaete chrysosporium*; Gold and Alic, 1993), or may be assigned to a post-translational modification. Clarification of this point is subject of future research.

The great overall amino acid similarity to versatile peroxidases of *Pleurotus eryngii* indicates that the β,β-carotene degrading enzyme from *L. irina* belongs to an unusual class of heme peroxidases. *P. eryngii* versatile peroxidases have been reported to share catalytic properties of lignin peroxidase and manganese peroxidase. They oxidize a wide variety of phenols and non-phenolic aromatic compounds either directly, similar to horseradish peroxidase, or indirectly through Mn^{3+} formed from Mn^{2+} like manganese peroxidases (Ruiz-Duenas *et al.*, 1999, 2001; Gomez-Toribio *et al.*, 2001). Further versatile peroxidases have been described from the white rot fungi *Pl. pulmonarius* (Camamero *et al.*, 1996), *P. ostreatus* (Sarkar *et al.*, 1997), and *Bjerkandera adusta* (Heinfling *et al.*, 1998). They are well known for degrading lignin, cellulose, hemicellulose, and numerous low molecular weight compounds including many xenobiotics. Nevertheless, the cleavage of β,β-carotene to norisoprenoidic flavor compounds by a fungal peroxidase has not been reported before.

The molecular mass of mature *L. irina* versatile peroxidase of ~50.5 kDa, as determined by means of size exclusion chromatography and by SDS-PAGE, differs considerably from the 34.6 kDa calculated from the deduced amino acid sequence. A high glycosylation degree may explain these findings. Ruiz-Duenas *et al.* (1999) derived a carbohydrate content of 5–7% for the *P. eryngii* enzymes from MALDI-TOF experiments. Optimal degradation of β,β-carotene occurred at pH values close to the isoelectric point of the *L. irina* versatile peroxidase (3.75). Possibly, binding of the non-polar substrate β,β-carotene is favored at maximum enzyme hydrophobicity.

Peroxidases usually require H_2O_2 or hydroperoxides for their catalytic activity. Peroxides may be generated by several direct or indirect enzymatic mechanisms. Direct reduction of O_2 to H_2O_2 is catalyzed *e.g*. by extracellular aryl alcohol oxidase, which has been identified in the culture supernatant of *L. irina* (Zorn *et al.*, unpublished results). Surprisingly, the oxidative cleavage of β,β-carotene was also observed with the purified enzyme in

transformation media essentially free of peroxides. Trace amounts of H_2O_2, initiating the peroxidation cycle of the enzyme, may be generated *via* autoxidation. Organic acids, hydroquinones, NADH, and thiols have been shown to support peroxidase activity in the absence of exogenous H_2O_2 (Kuan and Tien, 1993; Gomez-Toribio *et al.*, 2001).

Data on the oxidative degradation of carotenoids by peroxidases are rather scarce. Kanner and Mendel (1977) identified a carotenoid bleaching enzyme in aqueous paprika (*Capsicum annuum* L.) extracts. After partial purification, it exhibited typical characteristics of plant peroxidases. Commercial soybean and horseradish peroxidases, lipoxygenases, and catalases were tested for their potential to bleach flour. Partial degradation of β,β-carotene was observed with horseradish and soybean peroxidase as well as with lactoperoxidase. Addition of H_2O_2 enhanced the activity of lactoperoxidase and horseradish peroxidase, but was not essential for the carotenoid bleaching (Gelinas *et al.*, 1998). The oxidative cleavage of the vinylic carbon-carbon double bond of styrene was catalyzed by a *Coprinus cinereus* peroxidase (Tuynman *et al.*, 2000). Mechanistic considerations included several possible intermediates of peroxidases in the oxidation of alkenes: a concerted oxygen transfer, a metallooxetane intermediate, a radical cation, a cation, and a radical intermediate.

A potential mechanism for the cleavage of β,β-carotene between C9 and C10 by *L. irina* versatile peroxidase is depicted in Figure 6. Abstraction of a hydrogen atom from the allylic methyl group results in a resonance-stabilized carbon radical. Hydroperoxides are formed intermediately through reaction with oxygen, and subsequent Hock cleavage gives two carbonyl compounds (Krauch *et al.*, 1976). Dihydroactinidiolide, which contributes to the flavor of black tea, may be formed as a secondary metabolite *via* epoxidation of β-ionone to epoxy-5,6-β-ionone (Bosser *et al.*, 1995).

The rapid degradation of β,β-carotene, along with the disposal of potent flavor compounds, makes the versatile peroxidase of *L. irina* an interesting tool for biotechnological flavor production as well as for laundry applications.

Materials and Methods

General

The *Lepista irina* strain (CBS 458.79) was obtained from the Dutch 'Centraalbureau voor Schimmelcultures' (Baarn, The Netherlands). Due to the light and heat sensitivity of carotenoids all β,β-carotene-containing solutions were freshly prepared before use. All cultivations were performed in the absence of light and standard sterile techniques were applied. Quantitative data represent average values of duplicate analyses.

Chemicals

The constituents of nutrient media were purchased from Merck (Darmstadt, Germany). β,β-Carotene and Tween 40 (checked for the absence of peroxides before use) were obtained from Fluka/Sigma (Taufkirchen, Germany). Solvents were provided by BASF (Ludwigshafen, Germany) and Baker (Deventer, The Netherlands). All solvents were distilled before use.

Biotransformation of β,β-Carotene

L. irina was maintained on a β,β-carotene-containing growth agar as described previously (Zorn *et al.*, 2003). For preparation of precultures, 14 mm diameter agar plugs from the leading mycelial edge were transferred into 100 ml of standard nutrition solution (30 g l^{-1} glucose ×1 H_2O; 4.5 g l^{-1} asparagine×1 H_2O; 1.5 g l^{-1} KH_2PO_4; 0.5 g l^{-1} $MgSO_4$; 3.0 g l^{-1} yeast extract; 15 g l^{-1} agar agar; 1 ml l^{-1} trace element solution containing Cu, Fe, Zn, Mn, and EDTA; pH adjusted to 6.0) and homogenized using an Ultra Turrax (Janke & Kunkel, Staufen, Germany). After cultivation for two weeks at 24 °C and 150 rpm, the cultures were homogenized and 20 ml of the precultures were transferred into 500 ml Erlenmeyer flasks containing 250 ml of fresh standard nutrition solution. After 10 days, the fungal mycelium was separated from the growth medium by centrifugation (3300 *g*, 5 °C, 20 min). One hundred ml of cell-free medium of *L. irina* were mixed with 2.0 mg of solubilized β,β-carotene and incubated at 24 °C and 100 rpm. For kinetic investigations, the reaction was stopped after 0.5, 1, 2, 3, and 6 h, respectively, by addition of 2-mercaptoethanol (40 µl). Remaining β,β-carotene and metabolites thereof were extracted three times with pentane/dichloromethane (1:1 v/v). The organic phases were combined, washed with saturated $NaHCO_3$ solution, dried over Na_2SO_4 and concentrated to 5 ml using a Vigreux column. Prior to the extraction, α-terpineol was added as an internal standard. Biodegradation of β,β-carotene was monitored by means of HPLC/DAD, whereas the formation of volatiles was observed by means of GC/FID and GC/MS as described below.

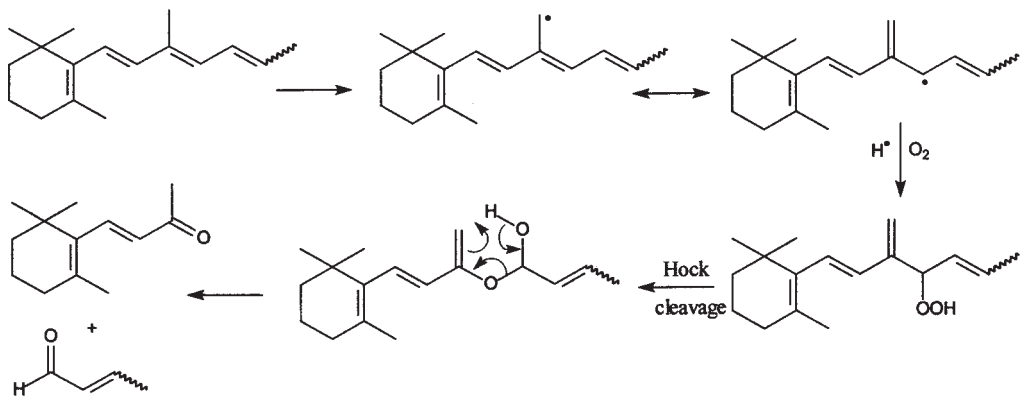

Fig. 6 Possible Reaction Mechanism of the Oxidative Cleavage of β,β-Carotene to β-Ionone (**3**) and β-Apo-10'-carotenal (**5**).

Enzyme Characterization

An enzyme assay (a modification of an assay of Ben Aziz *et al.*, 1971), was developed according to an approved method (Zorn *et al.*, 2003). Shortly, the time-dependent decrease of absorbance of an aqueous β,β-carotene emulsion was monitored at 450 nm using a thermostatable spectral photometer. Applicability of the test was checked by diluting aliquots of the enzyme sample with buffer solution (0.1 M citrate/phosphate buffer, pH 5.5). A linear correlation between activity and sample amount was found. For determination of the temperature optimum, 0.1 ml of β,β-carotene solution were added to 1.5 ml of cell-free growth medium of *L. irina*. The time-dependent decrease of absorbance was monitored at varying temperatures.

For determination of the pH optimum, cell-free medium of *L. irina* was concentrated 10-fold by ultrafiltration, using Centricon Plus-80 ultra filtration modules (exclusion limit 30 kDa; Millipore, Bedford, USA). For each measurement 0.2 ml of the concentrated retentate were diluted with 1.3 ml of buffer solutions of varying pH. 0.1 ml of β,β-carotene stock solution were added to each cuvette and the decrease in absorbance was monitored photometrically at 450 nm.

Protein Concentration

The protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin as a standard.

Electrophoresis

SDS-PAGE SDS-Page was performed by the method of Laemmli (1979) with 4% (w/v) polyacrylamide in the stacking gels and 14% (w/v) polyacrylamide in the resolving gels. Proteins were stained with 0.1% (m/v) Coomassie Brillant Blue G-250. A 'Low Molecular Weight Electrophoresis Calibration Kit' (Pharmacia Biotech, Uppsala, Sweden) was used for the preparation of a calibration curve for determination of molecular masses.

Isoelectric Focussing Mycelium-free culture supernatant was concentrated by ultra filtration (~2 mg ml^{-1} total protein), desalted, and subjected to isoelectric focussing polyacrylamide gel electrophoresis (IEF-PAGE) with immobilized pH gradient. Electrophoresis conditions: gel dimensions 12.5 cm × 12.5 cm × 0.3 mm; pH range 3 – 10; voltage 2000 V, 3 mA, 6 W, 3500 Vh; sample volume 10 µl.

The samples were applied twice, laterally reversed on both sides of the IEF gel. For detection, the gel was cut concentric and one half was subjected to Coomassie Blue staining and 'activity bleaching', respectively. Fifty ml of β,β-carotene solution (0.01% m/v + 1% m/v Tween 40), 15 ml buffer solution (7 mM citric acid, 6 mM Na_2HPO_4/NaH_2PO_4; pH 3.5), 100 µl trace element solution (containing Fe-, Cu-, Zn-, and Mn-ions), and 0.7 g agarose were mixed to give an orange coloured agarose gel of 2 mm thickness for the destaining test. One half of the IEF gel was covered with this carotene agar, pinned down and incubated at 34 °C for 1.5 hours. To ensure the correct assignment of protein bands, the decoloured spots were marked on the IEF gel by a pinprick and, after removal of the carotene agar, the gel was additionally stained by Coomassie Blue.

Enzyme Purification by Fast Protein Liquid Chromatography (FPLC)

All purification steps were performed in a cooling chamber at 6 °C. A Biologic Duoflow™ FPLC system (Bio-Rad, Hercules, USA) was employed for the chromatographic protein separation. The protein concentration of the eluate was monitored at λ=280 nm and enzyme activity was determined in all protein containing fractions.

Supernatant was collected from the submerged cultures after 10 days, separated from the mycelium by centrifugation, and concentrated 10-fold by ultrafiltration (see above). The retentate was equilibrated against the start buffer of hydrophobic interaction chromatography (HIC).

Hydrophobic Interaction Chromatography (HIC)

Separation was achieved on a 'HIC Phenyl high sub' column (20 ml, Amersham Pharmacia). An aqueous solution (pH 3.5) of citric acid (7 mM) and disodium hydrogenphospate/sodium dihydrogenphosphate (6 mM), containing ammonium sulfate (1 M) served as start buffer. The enzyme was eluted by changing to elution buffer (analogous to the start buffer, without ammonium sulfate) after 15 minutes. The flow rate was 4 ml min^{-1} and fractions were collected every minute. The active fractions of five HIC runs were pooled, concentrated by ultrafiltration (Ultrafree 4, exclusion limit 10 kDa; Millipore, Schwalbach, Germany), and equilibrated against the IEC start buffer.

Ion Exchange Chromatography (IEC)

A 'HiPrep 16/10 DEAE FF' column (20 ml, Amersham Pharmacia) was used for ion exchange chromatography. Start buffer was an aqueous solution of 7 mM citric acid and 6 mM disodium hydrogenphospate/sodium dihydrogenphosphate (pH 4.5). The enzyme was eluted by changing the elution buffer to citric acid, disodium hydrogenphospate/sodium dihydrogenphosphate (pH 3.5) after 25 min. The flow rate was 4 ml min^{-1}, fractions were collected every minute. The active fractions of three IEC runs were combined and concentrated by ultra filtration (see above).

Size Exclusion Chromatography (SEC)

A 'Superdex 200 HR 10/30' column (Amersham Pharmacia) with a bed volume of 24 ml and a separation range of 10 to 600 kDa was used. The elution buffer consisted of 35 mM citric acid and 30 mM disodium hydrogenphospate/sodium dihydrogenphosphate (pH 3,5; flow rate 0.5 ml min^{-1}). Fractions were collected every two minutes. A calibration curve was prepared with reference proteins (HMW and LMW Gel Filtration Calibration Kit, Pharmcia Biotech) for calculating molecular masses.

ESI-MS/(MS)

Spots from 1D gels were excised and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. A QTof II mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ion source and gold-coated capillaries (Protana, Odense, Denmark) was used for electrospray MS of peptides. For collision-induced dissociation experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (collision energy 25 – 30 eV for optimal fragmentation). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. The acquired MS/MS spectra were enhanced (Max. Ent. 3, Micromass) and used for the *ab initio* sequencing of tryptic peptids.

cDNA Synthesis and PCR-Screening

Cell disruption was achieved by grinding mycelium (160 mg, after 10 culture days) under liquid nitrogen. For isolation of total

RNA, a silica gel-based membrane (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) was applied. Integrity of the RNA was checked by denaturing formaldehyde agarose gel electrophoresis and ethidium bromide staining. A cDNA library was constructed in the λTriplEx2 vector according to the manufacturer's instructions (SMART™ cDNA library construction kit, BD Biosciences, Heidelberg, Germany). Superscript II (Gibco-BRL, Karlsruhe, Germany) RNase H- point mutant MMLV reverse transcriptase was used for first strand synthesis. The recombinant λ phages were packaged with Gigapack™ III Gold Packaging Extract (Stratagene, La Jolla, USA). The titer of the *L. irina* cDNA library was 5×10^6 pfu ml^{-1} before and 6×10^9 pfu ml^{-1} after amplification. λ-DNA was purified from liquid cultures using an optimized PEG precipitation step, followed by anion exchange chromatography (Lambda Kit, Qiagen). Advantage cDNA Polymerase Mix (BD Biosciences) and HotStarTaq Polymerase (Qiagen) were used for PCR amplification of *L. irina* versatile peroxidase cDNA. Primer construction was performed with the assistance of the primer3 algorithm (Rozen and Skaletsky, 2000).

DNA Sequencing

DNA was sequenced by the chain termination method (Sanger *et al.*, 1977) using a BigDye terminator cycle sequencing kit on an ABI Prism 310 Genetic Analyzer (PE Applied Biosystems, Foster City, USA).

Nucleotide Sequence Accession Number

The sequence of vsp1 was deposited at the EMBL Nucleotide Sequence Database under the accession number AJ515245.

Gas Chromatography/Mass Spectrometry (GC/MS)

A Hewlett-Packard HP 5890 (Waldbronn, Germany) gas chromatograph equipped with an on-column injector (40 °C) was directly coupled to a HP 5989 mass spectrometer. Separation of volatiles was achieved on a CP-5 CB fused silica column (30 m×0.32 mm i.d., film thickness 0.25 µm; Varian, Middelburg, The Netherlands), using helium as carrier gas (3.2 ml min^{-1}). The temperature was held at 50 °C for 5 min and then increased to 250 °C at a rate of 4 °C min^{-1}. The temperature of the ion source was 250 °C, the electron energy for all EI mass spectra was 70 eV. Degradation products of β,β-carotene were identified by comparing their Kovats indices and mass spectra with published data (Hohler, 1986; Krammer *et al.*, 2002) and, if available, with authentic reference materials.

Gas Chromatography/Flame Ionization Detector (GC/FID)

A Trace GC 2000 (Thermoquest, Mainz, Germany), equipped with a split-splitless injector (220 °C) and a flame ionization detector (250 °C) was applied for quantitative analyses. Separation of volatiles was achieved on a DB 5 column (30 m×0.25 mm i.d., film thickness 0.25 µm; J & W, Folsom, USA). Hydrogen was used as carrier gas at a flow rate of 2.1 ml min^{-1} and the same temperature program was used as for GC/MS. Quantitative data were determined using α-terpineol as the internal standard.

High Performance Liquid Chromatography with Diode Array Detection (HPLC/DAD)

For HPLC separation, a Nucleosil 120–5 analytical column (250×4 mm; Macherey & Nagel, Düren, Germany) with 5 µm C18 reversed phase material including a pre column (C18, 10×4 mm, 5 µm) was used. The mobile phase consisted of mixtures of methanol/water (containing 10 mM ammonium acetate) (70:30 v/v) (A) and methanol/dichloromethane (80:20 v/v) (B), starting with 100% A, followed by a gradient to obtain 100% B after 15 min, and isocratic 100% B from 15 to 45 min at a flow rate of 0.6 ml min^{-1}. The injection volume was 20 µl. Detection was performed with an UV/Vis-photodiode array detector MD 910 (Jasco, Groß-Umstadt, Germany).

HPLC-Mass Spectrometry

HPLC-MS-spectra were recorded on a VG Platform II (Micromass) in the $APcI^+$ mode, using the following settings: Corona: 3.2 kV, HV lens: 0.5 kV, Cone: 30 V, Source: 120 °C, Probe 350 °C.

UV/VIS-Spectroscopy

Absorption spectra were recorded using a Perkin Elmer Lambda 12 (Überlingen, Germany) spectral photometer equipped with thermostatable cell holder and magnetic stirrer.

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