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Generation of Norisoprenoid Flavors from Carotenoids by Fungal Peroxidases

Kateryna Zelena,[†] Björn Hardebusch,[†] Bärbel Hülsdau,[†] Ralf G. Berger,[†] and Holger Zorn*[,§]

[†]Leibniz Universität Hannover, Institut für Lebensmittelchemie, Callinstratsse 5, D-30167 Hannover, Germany, and [§]Justus-Liebig-Universität Giessen, Institut für Lebensmittelchemie und Lebensmittelbiotechnologie, Heinrich-Buff-Ring 58, D-35392 Giessen, Germany

To biotechnologically produce norisoprenoid flavor compounds, two extracellular peroxidases (MsP1 and MsP2) capable of degrading carotenoids were isolated from the culture supernatants of the basidiomycete *Marasmius scorodonius* (garlic mushroom). The encoding genes were cloned from genomic DNA and cDNA libraries, and databank homology searches identified MsP1 and MsP2 as members of the so-called "DyP-type" peroxidase family. Wild type enzymes and recombinant peroxidases expressed in *Escherichia coli* were employed for the release of norisoprenoids from various terpenoid precursor molecules. Carotenes, xanthophylls, and apocarotenals were subjected to the enzymatic degradation. Released volatile products were characterized by GC-FID and GC-MS, whereas nonvolatile breakdown products were analyzed by means of HPLC-DAD and HPLC-MS. C13 norisoprenoids together with C10 products proved to be the main volatile degradation products in each case.

KEYWORDS: Basidiomycete; peroxidase; carotenoids; norisoprenoid flavors

INTRODUCTION

In plants and fruits, numerous apocarotenoids are derived from an enzyme-catalyzed excentric cleavage of the polyene chains of carotenes and xanthophylls. Many of these cleavage products, so-called norisoprenoids, and especially the carbon 13 compounds, act as potent flavor compounds. β-Ionone, a molecule first found in the Bulgarian rose, has an odor threshold of 0.007 ppm (*1*), and β-damascenone, another important component of rose scents, is one of the most potent flavor-active organic molecules (*2*). The first carotenoid cleaving plant enzyme characterized on a molecular level was *AtCCD1* (*Arabidopsis thaliana* carotenoid cleavage dioxygenase 1) from *A. thaliana*. When expressed in *Escherichia coli*, the recombinant enzyme cleaved several carotenoids, including β-carotene, zeaxanthin, lutein, and violaxanthin symmetrically at the 9,10 and 9′,10′ positions of the polyene backbone. A C_{14} dialdehyde and two C_{13} norisoprenoid products were identified as reaction products (*3*). Convincing evidence for a dioxygenase mechanism of *AtCCD1* has been obtained from labeling experiments using $H_2{}^{18}O$ and $^{18}O_2$ (*4*). In the meantime, enzymes with properties similar to those of *AtCCD1* have been characterized from numerous plants, for example, tomato (*Lycopersicon esculentum*), crocus (*Crocus sativus*), petunia (*Petunia hybrida*), and wine (*Vitis vinifera* L.) (*5*).

The occurrence of norisoprenoids in their producer plants is typically restricted to trace amounts, and their extraction is often expensive and laborious. Thus, biotechnological processes copying natural carotenoid degradation have been envisaged. The best investigated approaches comprise the so-called co-oxidation with the lipoxygenase/linoleic acid system, the release of bound precursors by means of glycosidases, and the use of plant cell cultures. In the co-oxidation, the carotenoids are oxidized by free radical species generated from essential fatty acids by lipoxygenase catalysis (*6*). As none of these former approaches could be commercialized so far, a biotic carotenoid degradation by fungal enzymes was considered. In their natural environment, fungi are in a close contact with various carotenes and xanthophylls, and they thus should dispose of the enzymatic tools to degrade them. A first carotenoid cleaving fungal enzyme was purified and cloned from *Pleurotus eryngii*, which was erroneously named *Lepista irina* in the original publication (*7*). Only recently, two extracellular enzymes (MsP1 and MsP2) capable of degrading carotenoids have been purified from culture supernatants of the basidiomycete *Marasmius scorodonius* (garlic mushroom). The genes encoding MsP1 and MsP2 were cloned and sequenced from genomic DNA and cDNA libraries, and databank homology searches identified the enzymes as members of the so-called "DyP-type" peroxidase family (*8*). The aim of the present investigation was to open new biotechnological routes to natural flavor compounds derived from carotenoids. To that end, various carotenes, xanthophylls, and apocarotenals were isolated from plant matrices and subjected to enzymatic degradation by wild type and recombinant MsP1 and MsP2. Volatile and nonvolatile cleavage products were characterized.

MATERIALS AND METHODS

The *M. scorodonius* strain (CBS 137.86) was obtained from the Dutch "Centraalbureau voor Schimmelcultures", Baarn, The Netherlands. Production and purification of wild type enzymes

*Corresponding author (telephone +49 641-99 34 900; fax +49 641-99 34 909; e-mail holger.zorn@lcb.chemie.uni-giessen.de).

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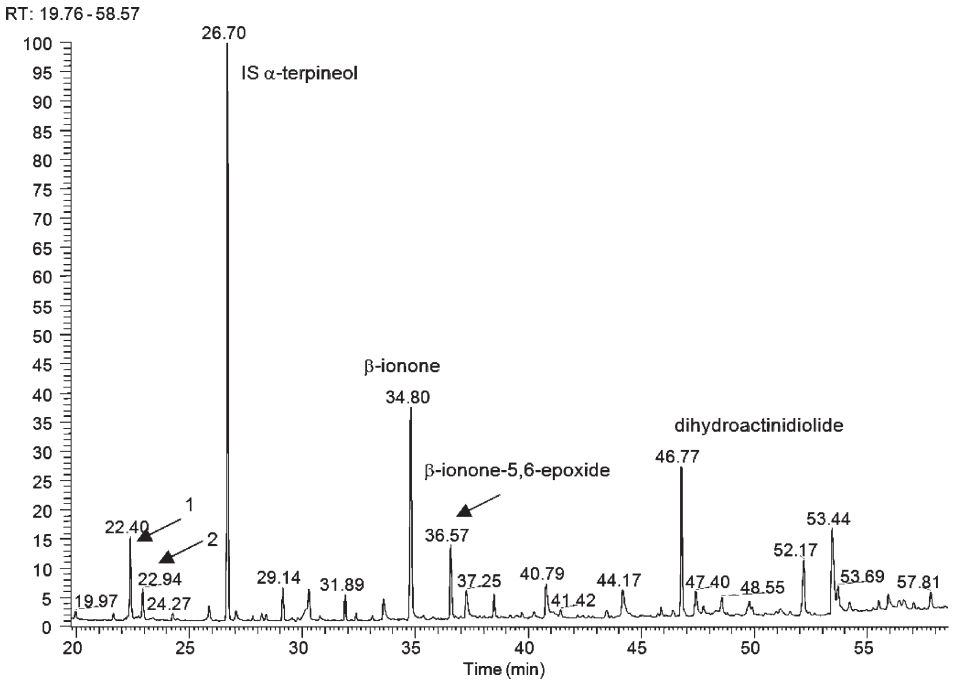

Figure 1. Cleavage of β-carotene to flavor compounds (GC-MS chromatogram; CW 20): 1 = 2,6,6-trimethylcyclohexanone; 2 = β-cyclocitral.

were performed as described in ref *8*, and recombinant MsP2 was produced in cultures of *E. coli* according to the method of Zelena et al. (*9*).

Substrates. β-Apo-8′-carotenal and β-apo-12′-carotenal were obtained from BASF (Ludwigshafen, Germany). Lycopene and zeaxanthin were donated from DSM (Delft, The Netherlands), and β-carotene was purchased from Fluka (Seelze, Germany). Violaxanthin and neoxanthin were extracted from spinach and purified by preparative HPLC. Therefore, 250 g of spinach was extracted with 3×250 mL of an acetone/saturated $NaHCO_3$ solution (4:1, v/v) in a kitchen blender. The combined extracts were filtered through a Büchner funnel, and the solvent was removed at 40 °C and 500 mbar in a rotary evaporator. After saturation of the aqueous phase with NaCl, the xanthophylls were extracted with 3 × 100 mL of diethyl ether. The combined organic extracts were dried over anhydrous sodium sulfate and concentrated, and ether residues were removed under a stream of nitrogen. The resulting extract was redissolved in 5 mL of acetone, filtered through a 0.45 μm membrane filter, and subjected to semipreparative HPLC.

For semipreparative HPLC separation, a Nucleosil 120-5 column (250 × 16 mm; Macherey & Nagel, Düren, Germany) with 5 μm C18 reversed phase material was used. The mobile phase consisted of mixtures of methanol/water (80:20 v/v) (A) and ethyl acetate (B), starting with 80% A, followed by a gradient to obtain 23% B after 5 min, 50% B after 40 min, and 100% B after 50 min at a flow rate of 5.0 mL/min. The injection volume was 500 μL. Detection was performed with a UV-1570 detector (Jasco, Gross-Umstadt, Germany) at 450 nm. The respective violaxanthin- and neoxanthin-containing fractions of several runs were combined and concentrated in a rotary evaporator at 200 mbar and 40 °C. The aqueous residue was extracted with ethyl acetate, and the organic extract was filled to a final volume of 20 mL. The yields of neoxanthin and violaxanthin were 18.5 and 38.8 mg/kg, respectively.

Lutein was released by saponification of marigold (*Tagestes erecta*) oleoresin. To this end, 10 g of oleoresin was mixed with 50 mL of diethyl ether and saponified at room temperature overnight with methanolic potassium hydroxide (10% w/v). For complete removal of alkali, the solution was washed with 3 × 50 mL of water; the organic layer was dried over anhydrous sodium sulfate, filtered through a folded filter, and evaporated to dryness. The extract was stored at −18 °C under nitrogen until use.

UV−Vis Spectroscopy. Absorption spectra were recorded using a Lambda 12 (Perkin-Elmer, Überlingen, Germany) spectral photometer equipped with thermostatable cell holder and magnetic stirrer. Calculation of the substrate concentration was performed by means of UV−vis spectrophotometry using the parameters (solvents and wavelengths) suggested by Britton et al. (*10*).

Biotransformation. Substrate emulsions (0.01%) were prepared as described in ref *11*. The biotransformation was performed with 300 μg of substrate in a total volume of 15 mL of sodium acetate buffer (50 mM, pH 5.0) for 60 min at 27 °C (150 rpm) and initiated by the addition of wild type or recombinant enzyme preparation (12 mU) and 5 μL of 20 mM H_2O_2 solution. For the blanks, the enzyme samples were heat inactivated (100 °C, 20 min) prior to the reaction.

Product Purification and Identification. The degradation products were purified by SPE (Chromabond C_{18}, Macherey & Nagel) and identified by GC-MS by comparing their Kovats indices and mass spectra with published data. Quantification was performed by GC-FID using (+)-α-terpineol (1.42 mM) as an internal standard.

After further purification of the extract by flash chromatography on silica 60 (60−200 mesh, Merck, Darmstadt, Germany; elution with pentane/diethyl ether 80:20, v/v), nonvolatile breakdown products were analyzed by HPLC-DAD and HPLC-MS.

Gas Chromatography−Flame Ionization Detector (GC-FID). High-resolution GC-FID using a polar phase was performed on a Trace GC equipped with a DB-Wax column (30 m×0.32 mm i.d., film thickness = 0.25 μm, SGE, Griesheim, Germany). A Fisons GC 8000, equipped with a split−splitless injector (230 °C) and a flame ionization detector (300 °C), was applied for quantitative analyses on a nonpolar phase. 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, CA). Hydrogen was used as carrier gas at a flow rate of 2.5 mL/min, and the same temperature program was used as for GC-MS.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analysis using a polar phase was conducted on a Fisons GC 8000 equipped with a (polyethylene glycol) ZB-Wax (30 m×0.32 mm i.d., film thickness = 0.25 μm, Phenomenex, Torrance, CA) column connected to a Fisons MD800 mass selective detector. GC-MS analysis using a nonpolar phase was performed on an HP5890 series II GC equipped with a ZB-5MS (30 m × 0.32 mm i.d., film thickness = 0.25 μm, Varian, Palo Alto, CA) column connected to an HP quadrupole mass spectrometer 5989. Both GC-MS instruments were operated at 70 eV in the EI mode over the range of 33−500 amu. Helium was used as the carrier gas at a flow rate of 3.1 mL/min (polar phase) or 3.3 mL/min (nonpolar phase), respectively. The injection volume was 1 μL cool on-column (40 °C). The oven temperature was held at 40 °C for 3 min, raised at 5 °C/min to a final temperature of

Table 1. Tentatively Identified Volatile Degradation Products of Carotenes and Xanthophylls

substrate	degradation product	yield (mol %) (mg L^{-1})	Kovats index	GC-MS (*m*/*z*)
β-carotene	β-ionone[c]	7.9 (0.6)	1911[a]	192 ($M^{\bullet+}$), 177 (100), 135, 107, 105
	β-ionone-5,6-epoxide	1.3 (0.1)	1964[a]	208 ($M^{\bullet+}$), 135, 124, 123 (100)
	dihydroactinidiolide	7.0 (0.5)	2321[a]	180 ($M^{\bullet+}$), 137, 111 (100), 110, 109
	β-cyclocitral[c]	1.5 (<0.1)	1595[a]	152 ($M^{\bullet+}$), 137, 123, 109, 67 (100)
	2-hydroxy-2,6,6-trimethylcyclohexanone	2.5 (0.1)	1583[a]	156 ($M^{\bullet+}$), 128, 110, 95, 71 (100)
lutein	3-hydroxy-α-ionone	11.0 (0.8)	1627[b]	208 ($M^{\bullet+}$), 147, 125, 124, 109 (100)
	3-hydroxy-β-ionone	6.3 (0.5)	1677[b]	208 ($M^{\bullet+}$), 193 (100), 175, 147, 131
zeaxanthin	3-hydroxy-β-ionone	5.7 (0.4)	1677[b]	208 ($M^{\bullet+}$), 193 (100), 175, 147, 131
	3-hydroxy-β-cyclocitral	traces	2346[a]	168 ($M^{\bullet+}$), 135 (100), 121, 107, 91
violaxanthin, neoxanthin	3-hydroxy-β-ionone-5,6-epoxide	6.9 (0.5) 6.8 (0.5)	1688[b]	224 ($M^{\bullet+}$), 125, 124, 123 (100), 109
lycopene	geranial	1.3 (<0.1)	1269[b]	152 ($M^{\bullet+}$), 136, 121, 107, 69 (100)
	6-methyl-5-heptene-2-one	7.2 (0.3)	1324[a]	126 ($M^{\bullet+}$), 111, 108 (100), 93, 71

[a] M&N CW 20 M, 30 m × 0.32 mm i.d., 0.25 μm film thickness. [b] J&W DB 5, 30 m × 0.32 mm i.d., 0.25 μm. [c] Compound identified via reference standard.

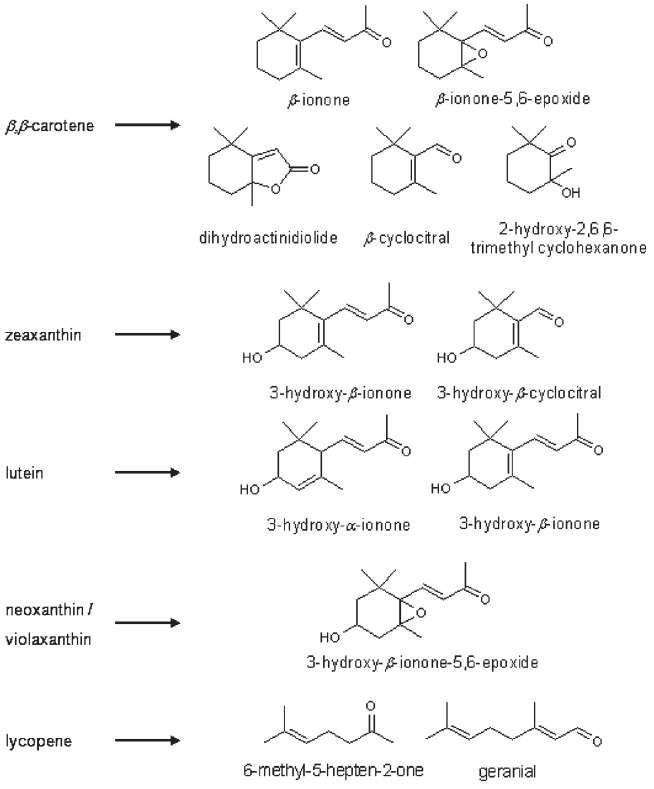

Figure 2. Norisoprenoid compounds released from carotenes and xanthophylls by *M. scorodonius* peroxidase catalysis.

240 °C (polar phase) or 280 °C (nonpolar phase), respectively, and held constant at the final temperature for 10 min. Linear retention indices (RIs) were calculated according to the Kovats method using *n*-alkanes (C_7−C_{28}) as external references. Mass spectral identification was completed by comparing spectra with commercial mass spectral databases Wiley, NIST, and LIBTX and by comparison with authentic reference standards. β-Ionone (95%) and α-terpineol (puriss.) were obtained from Fluka, and β-cyclocitral (>90%) was purchased from Sigma-Aldrich.

High-Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD). For HPLC separation, a Nucleosil CC 100-5 analytical column (250×4 mm; Macherey & Nagel) with 5 μm C18 reversed phase Nautilus material including a precolumn (C18, 10×4 mm, 5 μm) was used. The mobile phase consisted of mixtures of water/acetonitrile (0−4 min 50:50 v/v; 4−20 min 0:100 v/v; 20−60 min isocratic 0:100 v/v) at a flow rate of 1.0 mL/min. The injection volume was 20 μL. Detection was performed with a SPD-M6A UV−vis photodiode array detector (Shimadzu, Duisburg, Germany).

Table 2. Tentatively Identified Volatile Degradation Products of Apocarotenals

	substrate (mol %) (mg L^{-1})	
product	β-apo-8′-carotenal	β-apo-12′-carotenal
β-ionone[a]	5.4 (0.5)	3.2 (0.4)
β-ionone-5,6-epoxide	1.9 (0.2)	1.0 (0.1)
dihydroactinidiolide	2.1 (0.2)	2.2 (0.2)
2-hydroxy-2,2,6-trimethylcyclohexanone	1.4 (0.1)	traces
β-cycolcitral[a]	1.6 (0.1)	2.4 (0.2)

[a] Compound identified via reference standard.

HPLC−Mass Spectrometry. HPLC-MS spectra were recorded on a LCMS-QP8000α system (Shimadzu) in the $APcI^+$ mode, using the mobile phase and flow rate described above.

RESULTS AND DISCUSSION

Various carotenes, xanthophylls, and apocarotenals were subjected to enzymatic degradation by the *M. scorodonius* peroxidase. The biotransformation was performed at pH 5.0, where MsP1 exhibited maximal catalytic activity (*11*). Although the dimeric enzyme MsP1 turned out to be rather thermostable up to temperatures of about 65 °C (*12*), a reaction temperature of 27 °C was chosen to avoid losses of the released volatiles. Under these conditions, all of the substrates were readily degraded within 60 min, and C13 norisoprenoids together with C10 products proved to be the main volatile degradation products in each case (**Figure 1** and **Table 1**). None of the degradation products was detected when heat-inactivated enzyme samples were employed as blanks.

A potential mechanism for the carotenoid cleavage between C9 and C10 could start with the abstraction of a hydrogen atom from the allylic methyl group, resulting in a resonance-stabilized carbon radical. Hydroperoxides may be formed intermediately through reaction with oxygen, and subsequent Hock cleavage would yield two carbonyl compounds (*7*).

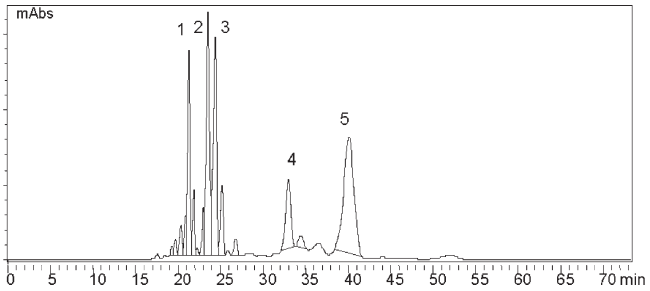

Figure 3. Enzymatic cleavage of β-carotene by extracellular enzymes of *M. scorodonius*; HPLC-MS chromatogram (1 = β-apo-14′-carotenal; 2 = β-apo-12′-carotenal; 3=β-apo-10′-carotenal; 4=β-carotene-monoepoxide; 5=β-carotene-5,6-epoxide).

Table 3. Nonvolatile Degradation Products of Carotenes and Xanthophylls Tentatively Identified by HPLC-DAD and HPLC-MS Analyses

substrate	product retention time (min)	$[M + H^+]$	tentative identifiaction
β-carotene	21.3	311	β-apo-14′-carotenal
	23.5	351	β-apo-12′-carotenal
	24.4	377	β-apo-10′-carotenal
	33.0	553	β-carotene-monoepoxide
	40.1	553	β-carotene-5,6-epoxide
lutein/zeaxanthin	15.5	327	3-hydroxy-apo-14′-carotenal
	19.5	367	3-hydroxy-apo-12′-carotenal
	20.8	393	3-hydroxy-apo-10′-carotenal
	23.5	433	3-hydroxy-apo-8′-carotenal
	40.9	585	lutein/zeaxanthin epoxide
violaxanthin/ neoxanthin	12.4	343	3-hydroxy-β-apo-14′-carotenal-5,6-epoxide
	16.5	383	3-hydroxy-β-apo-12′-carotenal-5,6-epoxide
	17.7	409	3-hydroxy-β-apo-10′-carotenal-5,6-epoxide

The formation of β-ionone-5,6-epoxide may result from the cleavage of β-carotene-5,6-epoxide or from the epoxidation of released β-ionone. β-Carotene-5,8-epoxide probably represents the direct precursor of dihydroactinidiolide (*13*). 3-Hydroxy-α-ionone was obtained from the degradation of lutein, and 3-hydroxy-β-ionone was released from lutein and zeaxanthin. Analogously, 3-hydroxy-β-ionone-5,6-epoxide was formed from violaxanthin and neoxanthin (**Figure 2**). These compounds have been described as flavor precursors in, for example, carambola, nectarines, and tobacco (*14*, *15*). 3-Hydroxy-β-ionone-5,6-epoxide has been postulated to be the biosynthetic precursor of megastigma-7-ene-5,9-diol-3,6-epoxide in tobacco (*16*). With lycopene as a substrate, the flavor compounds 6-methyl-5-heptene-2-one and geranial were formed. Both compounds are important constituents of the flavor of tomatoes (*17*).

β-Apo-8′-carotenal and β-apo-12′-carotenal were selected as representatives for nontetraterpenoid carotenes. The spectrum of volatile cleavage products generated by peroxidase treatment was dominated by β-ionone, dihydroactinidiolide, and β-cyclocitral and thus was comparable to that obtained with β-carotene as substrate (**Table 2**).

Nonvolatile breakdown products of carotenes and xanthophylls were separated and tentatively identified on the basis of their UV−vis spectra (*10*) and molecular masses by HPLC-DAD and HPLC-MS (**Figure 3** and **Table 3**). Although the nonvolatile carotenoid cleavage products listed above were detected in low parts per million concentrations only, all of the carotenoids were readily degraded under the experimental conditions chosen. Whereas the cleavage of zeaxanthin, lycopene, and neoxanthin amounted to about 30%, almost 60% of the β-carotene was degraded within 60 min under the same conditions. Considering the obtained yields of volatiles and the presence of only trace amounts of nonvolatile degradation products, there is still a significant gap in the mass balance. Further investigations will be necessary to close this gap and to fully understand the reaction pathway.

Data on carotenoid degradation by peroxidases are rather scarce. Kanner and Mendel (*18*) identified a carotenoid bleaching enzyme in aqueous paprika extracts, which showed typical characteristics of plant peroxidases. A carotenoid degrading peroxidative activity was found in solubilized thylakoid membranes of olives (*19*), and at least partial degradation of β-carotene was observed with soybean and horseradish peroxidase as well as with lactoperoxidase (*20*). Different from the highly selectively acting plant carotenoid cleavage enzymes, the peroxidase-catalyzed reaction resulted in a broader spectrum of cleavage products. When an enzyme model based on a ruthenium tetramesitylporphyrin catalyst was used to degrade β-carotene, similar product spectra were obtained (*21*, *22*).

In conclusion, the extracellular peroxidases of *M. scorodonius* efficiently degraded carotenoids to norisoprenoid flavor compounds. The H_2O_2 required for the catalytic activity of the peroxidases may be supplemented or generated in situ by the addition of glucose and glucose oxidase. Apart from the production of "bioflavors", the novel enzymes could become interesting tools in detergents and food-bleaching applications (*23*).

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