In vitro Study of the Carotenoid-cleavage Enzyme from *Staphylococcus pasteurii* TS-82 Revealed Substrate Specificities and Generation of Norisoprenoid Flavors

Ming-Ming Zhu, Shu-Lin Wang¹, Ming-Tao Fan*, and Jing Li

College of Food Science and Engineering, Northwest A&F University, Yangling, China

¹Agriculture and Animal Husbandry College, Qinghai University, Qinghai, China

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*Corresponding Author
Tel: +86-29-87092486
Fax: +86-29-87092486
E-mail: fanmt@nwsuaf.edu.cn

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Abstract Reactions of a crude enzyme extracted from *S. pasteurii* TS-82 to cleave carbon-carbon bonds in bicyclic and monocyclic carotenoid substrates were investigated. Dependencies of enzyme activities on processing temperature and pH were investigated and non-volatile and volatile breakdown products were characterized. The crude enzyme showed a maximum activity with zeaxanthin, followed in decreasing order by β -carotene, canthaxanthin, astaxanthin, and β -apo-8'-carotenal. The optimum pH value of the enzyme was 3.0 for both bicyclic and monocyclic substrates, whereas the optimum temperature of the enzyme was substrate specific at 60°C for C₄₀ carotenoids and 50°C for β -apo-8'-carotenal. Liquid Chromatography-Mass Spectra (LC-MS) and Gas Chromatography-Mass Spectra (GC-MS) indicated that the crude enzyme was able to catalyze substrates with cleavage at 9-10 and 9'-10' double bonds with C₁₃ norisoprenoids being the main volatile reaction products in each case. Astaxanthin is a major source for α , β -dihydro- β -ionone.

Keywords: *Staphylococcus pasteurii* TS-82, crude enzyme, carotenoids, aroma compounds

Introduction

Sea buckthorn (*Elaeagnaceae*) is a hardy, deciduous shrub with yellow or orange berries (1) rich in the nutritive compounds carotenoids, chlorophylls, steroids, fatty acids, vitamins, and flavonoids (2). More attention has recently been paid to commercial use and product development using these berries (3). However, the berries have a short shelf-life and are susceptible to physical damage and microbial contamination (4). Therefore, processing of sea buckthorn berries soon after harvest is important. Juice processing requires heat treatment and pasteurization (5), causing loss of some volatile fragrance compounds, while wine fermentation is usually carried out at normal temperatures, retaining most volatile fragrance compounds (6). Yet, a lingering problem for sea buckthorn wine is sourness and blandness. Sourness can be reduced to a reasonable level using chemical, physical, or biological approaches. Blandness can also be improved or modified through carotenoid degradation in sea buckthorn wine processing to produce aroma compounds.

Carotenoids are natural colorants that are responsible for attractive colors of several foods (7) and are also important substrates of some carotenoid-derived aroma compounds with low flavor thresholds that are important contributors to wine quality (8-10). β -Ionone and β -damascenone are important carotenoid-derived

aroma compounds with threshold values of 0.007 and 0.009 μ g/L in water (11,12). Formation of norisoprenoids as aroma-impact compounds via enzymatic reactions has been characterized from plants (13,14), insects, birds, and mammals (15), and also from microorganisms, such as fungi (16-18), proteobacteria (19) and photosynthetic bacteria, including cyanobacteria (20).

S. pasteurii TS-82 (GeneBank Accession No. KP171185) was previously isolated from sea buckthorn broth and confirmed to have an enzyme for cleavage of β -carotene to produce aroma compounds; however, the mechanism of carotenoid degradation and optimum conditions remain unknown. The aim of this study was to elucidate degradation of carotenoids by carotenoid-cleavage enzymes isolated from a fermented *S. pasteurii* medium based on enzymatic reaction products using LC-MS and GC-MS techniques, and to investigate the optimum temperature and pH for the crude enzyme.

Materials and Methods

Crude enzyme extraction The *S. pasteurii* TS-82 wild-type strain was cultured in a standard nutrient medium (15 g/L sucrose, 0.5 g/L K₂HPO₄, 0.5 g/L MgSO₄, 0.01 g/L FeSO₄, 0.5 g/L KCl, 3 g/L NaNO₃, and 2 g/L yeast extract, all reagents were analytical grade chemicals.) for

12 h at 37°C. Cells were removed by centrifugation (HC-3018R; Anhui USTC Zonkia Scientific Instruments Co., Ltd., Hefei, China) at 4°C, 11,180×g for 20 min and target enzyme extracts remained in supernatants. Clear, cell-free culture supernatants containing crude enzyme extracts were concentrated in a freeze dryer (Coolsafe 110-4; Gene Co., Ltd., Hong Kong, China) and loaded onto a Mono Q 10/100 chromatograph (10 µm, 10×100 mm) (GE, Fairfield, CT, USA) for primary purification. The target enzyme was collected and concentrated to a freeze-dried powder in a freeze dryer (Coolsafe 110-4; Gene Co., Ltd.).

Chemicals β-Apo-8'-carotenal, β-carotene, lycopene, zeaxanthin, canthaxanthin, astaxanthin, and β-ionone were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Methanol and acetonitrile (HPLC grade) were purchased from Tedia (Cincinnati, OH, USA). Methyl *tert*-butyl ether of HPLC grade (LC-20A; Shimadzu, Kyoto, Japan) was purchased from Aladdin (Shanghai, China). All other reagents used in this study were analytical grade chemicals.

Carotenoid preparation For *in vitro* assays, carotenoid substrates were applied at a concentration of 120 µM. Due to carotenoid light and heat sensitivities, all solutions were freshly prepared before use. For preparation of carotenoid micelles, substrates were dissolved in 10 mL of dichloromethane (analytical grade) and mixed with 1 g of Tween-80 (analytical grade), then dried in a vacuum centrifuge (SCANS40; Scanlaf, Labogene, Denmark) to produce a carotenoid-containing micelle. Residues were resuspended in ultrapure water and stored at 4°C prior to use.

Substrate specificity The substrate specificity of the crude enzyme was investigated using lycopene, β-apo-8'-carotenal, β-carotene, lycopene, zeaxanthin, canthaxanthin, and astaxanthin as substrates. Biotransformation was performed with 3 mL of a 120 µM substrate solution in a total volume of 10 mL of sodium acetate buffer (20 mM) at pH 5.0 initiated via addition of 120 µg of crude enzyme at 30°C water bath (HH2A; Kewei Co., Ltd., Beijing, China) for 30 min. A control solution was prepared under the same reaction conditions without addition of a crude enzyme. Reactions were stopped by adding 10 mL acetone. Lipophilic compounds were extracted according to the method established by Hoffmann *et al.* (19) with some modification. The carotenoids were extracted by adding two volumes of hexane, then standing for 5 min and the mixture was centrifuged at 2,795×g for 5 min at 4°C. The carotenoids remained in the supernatants, which were evaporated in the vacuum centrifuge (SCANS40; Scanlaf), and the residue was dissolved in 10 mL acetone. Substrate degradation was measured using High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) (LC-20A; Shimadzu). HPLC analyses were then performed using 20 µL of the extracts. A pre-packed reversed-phase C₁₈ column (5 µm, 4.6×250 mm, Shimadzu) was used for separation of the substrates at a flow rate of 1 mL/min.

Solvent A was a mixture of methanol, water, and methyl *tert*-butyl ether (30:10:1) (v/v). Solvent B was composed of methanol and methyl *tert*-butyl ether (1:1) (v/v). The HPLC gradient was performed as follows (defined as percentage of solvent B): 0-30 min, 0-100% (v/v); 30-35 min, 100%; 35-36 min, 100-0%; 36-51 min, 0%. The degradation of the substrates was measured with HPLC at 460 nm. All operations were performed under aluminum foil to avoid light.

Enzymatic activity was investigated based on recording of peak areas of each substrate and controls for 30 min at 460 nm. A decrease in substrate amounts before and after incubation was used for estimation of relative enzymatic activity. All experiments were performed in triplicate.

Determination of kinetic parameters The Michaelis-Menten constant (K_m) (21) and maximum rate of the reaction (V_{max}) were determined for reactions based on incubation of enzyme extracts in the presence of increasing 10-120 µM concentrations of the substrates β-apo-8'-carotenal, β-carotene, zeaxanthin, canthaxanthin, and astaxanthin. Enzymatic activity was monitored using addition of 120 µg of crude enzyme to the reaction mixture. The reaction was performed at pH 5.0 and 30°C for 10 min, then reaction was stopped by using 10 mL acetone and extracts were subjected to HPLC (LC-20A; Shimadzu) as described above. K_m and V_{max} values for different substrates were determined based on plotting activity data as a function of substrate concentration in a Lineweaver-Burk plot (21).

Influence of temperature on enzyme activity The effect of temperature on the carotenoid cleavage activity was evaluated using 5 carotenoid substrates. Enzymatic reaction mixtures contained 3 mL of a 120 µM substrate solution in a total volume of 10 mL of sodium acetate buffer (20 mM, pH 5.0) and a reaction initiated by addition of 120 µg of crude enzyme. Enzyme reaction mixtures were incubated at 30, 40, 50, 60, and 70°C for 30 min. A control solution was prepared under the same reaction conditions at temperatures of 30, 40, 50, 60, and 70°C without addition of a crude enzyme, respectively. Reaction mixtures were subjected to extraction and extracts were subjected to HPLC (LC-20A; Shimadzu) as described above. All experiments were performed in triplicate.

Influence of pH on enzyme activity The pH dependency of enzyme activity was also measured using 5 carotenoid substrates. Enzyme reaction mixtures contained 3 mL of a 120 µM substrate solution in a total volume of 10 mL of a buffer at different pH values of 2.0-3.5 for 20 mM glycine HCl, 4.0-5.5 for sodium acetate buffer, and 6.0 for sodium phosphate, with reactions initiated by addition of 120 µg of crude enzyme. A control solution was prepared under the same reaction conditions at different pH values 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, and 6.0 without addition of a crude enzyme. Enzymatic reaction mixtures and controls were incubated at 30°C water bath (HH2A; Kewei Co., Ltd.) for 30 min. Reaction mixtures were then subjected to extraction and extracts were subjected to HPLC (LC-20A;

Shimadzu) as described above. All experiments were performed in triplicate.

LC-MS identification of enzymatic non-volatile reaction products

Analysis was performed using HPLC (Dionex Ultimate 3000; Dionex Corp., Sunnyvale, CA, USA). Chromatographic separation was performed using a Hypersil GOLD C₁₈ column (2.1×50 mm, i.d., 5 μm) (Thermo Fisher, Waltham, MA, USA). Solvent A was a mixture of water and acetonitrile (95:5) (v/v). Solvent B was acetonitrile. The flow rate was 0.3 mL/min. The non-linear HPLC gradient was, given as percentage of solvent A, 0-20 min at 50-0% (v/v), 20-40 min at 0%, 40-41 min at 0-50%, and 41-47 min at 50%.

MS analysis was performed using a maXis Q-TOF mass spectrometer (Brooks Automation, Inc., Chelmsford, MA, USA). The atmospheric pressure ionization ion source was used in positive mode. The MS parameters were corona=4,000 nA, dry temperature=200, APCl heater=400, capillary=2.5 KV, and a mass range (m/z)=200-2,500.

GC-MS identification of volatile enzymatic reaction products

Degradation products of β-carotene and β-apo-8'-carotenal were extracted using solid phase micro extraction (SPME) (12,22). Zeaxanthin, canthaxanthin, and astaxanthin flavor products were extracted via addition of 10 mL of acetone and 2.5 g of NaCl. Carotenoids were dissolved in acetone and identified using GC-MS (Trace DSQ; Thermo-Finnigan, San Jose, CA, USA) based on comparison of mass spectra with previous values (17).

The GC-MS apparatus (Trace DSQ; Thermo-Finnigan) was equipped with a DB-WAX (60 M×0.25 mm×0.25 μm) fused silica capillary column (Agilent Inc., Santa Clara, CA, USA) for detection of products cleaved from β-carotene and β-apo-8'-carotenal, or with a DB-5MS (60 M×0.25 mm×0.25 μm) column (Agilent Inc.) for detection of products cleaved from zeaxanthin, canthaxanthin, and astaxanthin. GC-MS instruments were operated at 70 eV in EI mode over a range of 35-400 amu. Helium was used as a gas carrier at flow rate of 1 mL/min. The ion source temperature was 250°C. For the polar phase, the injection temperature was 250°C. The transfer line temperature was 230°C. The oven temperature was kept at 40°C for 2.5 min, increased to 200°C at 5°C/min, increased to a final temperature of 240°C at 10°C/min, then maintained at 240°C for 5 min. For the nonpolar phase, the injection volume was 1 μL into the column (80). The oven temperature was kept at 80°C for 2.5 min, increased to a final temperature of 280°C at 6°C/min, and kept at 280°C for 10 min.

Results and Discussion

Substrate specificity Crude enzymes from *S. pasteurii* TS-82 showed carotenoid-cleavage activities for degradation of the 6 different carotenoid substrates β-apo-8'-carotenal, β-carotene, lycopene, zeaxanthin, canthaxanthin, and astaxanthin. Carotenoid-cleavage enzymes have also been isolated directly from *Ischnoderma*

benzoinum and *Trametes versicolor* (18,23). These microorganism-derived crude enzymes displayed relatively broad substrate specificities (24).

Only a small amount of lycopene was converted by the crude enzyme *in vitro*, perhaps due to insufficient solubilization of lycopene in the process of producing a carotenoid-containing micelle. A similar result was obtained with *Sphingopyxis alaskensis* carotenoid cleavage oxygenase (SaCCO) and *Mycobacterium tuberculosis* carotenoid cleavage oxygenase (MtCCO) (19,25). In contrast, other substrate conversions could be observed visually based on color changes of reactants, and using HPLC graphs (Fig. 1A, 1B, and 1C).

A decrease in substrate amounts was used for estimation of the cleavage efficiency of different substrates catalyzed by the crude enzyme (Table 1). In 30 min assays, there was a difference in conversion among the 5 substrates. The conversion rate was more than 0.1 μmol for β-carotene, canthaxanthin, and astaxanthin, and approximately 0.2 μmol for zeaxanthin, and approximately 0.03 μmol of β-apo-8'-carotenal was converted in 30 min. Carotenoid cleavage activities were higher with C₄₀ carotenoid as a substrate than with β-apo-8'-carotenal. For *Nostoc* sp. PCC 7120 *in vitro* assays, a higher enzymatic activity was also observed using the C₄₀ carotenoid zeaxanthin than with β-apo-8'-carotenal (26). The preference of the crude enzyme for zeaxanthin may have been due to a higher solubility than for β-carotene, canthaxanthin, and astaxanthin. However, the reason for preference of the crude enzyme for bicyclic substrates over monocyclic substrates remains unclear.

Enzyme kinetics K_m and V_{max} values for different substrates are listed in Table 1. Zeaxanthin showed the lowest K_m value of 9.441 μM and the K_m value increased using less specific substrates. β-Carotene, canthaxanthin, astaxanthin, and β-apo-8'-carotenal showed 30, 42, 48, and 63% higher K_m values than zeaxanthin. Based on V_{max} and K_m values, the enzyme showed highest affinity toward zeaxanthin, followed in a decreasing order by β-carotene, canthaxanthin, astaxanthin, and β-apo-8'-carotenal, indicating that carotenoid cleavage activities were higher with C₄₀ carotenoids as a substrate than with β-apo-8'-carotenal.

Influence of temperature on enzyme activity A temperature profile showed an increase of enzyme activity as temperature increased from 30 to 60°C for C₄₀ carotenoids and from 30 to 50°C for β-apo-8'-carotenal. Then, as temperature continued to increase, the enzyme activity gradually decreased (Fig. 2A). The optimum temperature for the carotenoid-cleavage enzyme was 60°C for C₄₀ carotenoids and 50°C for β-apo-8'-carotenal. Optimum temperatures were 70°C for the enzymes with carotenoid cleavage activities from tea and pandan (27) and 65°C for the enzyme with carotenoid cleavage activities from *Pleurotus eryngii* (17). These optimum temperatures for enzymes with carotenoid cleavage activities indicated that the *S. pasteurii* TS-82 enzyme studied here can be an excellent alternative for use in the food industry, especially for thermal processes.

Fig. 1. LC-MS analysis of β -carotene cleavage products synthesized *in vitro* using a crude enzyme from *S. pasteurii* TS-82. (A) HPLC chromatogram and a photograph of the reaction mixture without the crude enzyme (control). (B) HPLC chromatogram and a photograph of the reaction mixture with the crude enzyme (30 min, 30°C, pH 5.0). (C) HPLC chromatogram and a photograph of the reaction mixture with the crude enzyme (30 min, 30°C, pH 4.0). (D-F) Mass spectra and structures of (D) product 1 (mutatochrome), (E) product 2 (β -carotene-5,6-epoxide), and (F) product 3 (β -carotene).

Table 1. K_m and V_{max} values of the crude enzyme toward different substrates for 10 min and *in vitro* cleavage efficiency of carotenoids for 30 min

Substrate	K_m ¹⁾ (μ M)	V_{max} ²⁾ (mM/min)	Substrate conversion ³⁾ (μ mol)
β -Carotene	9.441 $\pm$ 0.191	1.374 $\pm$ 0.012	0.1353 $\pm$ 0.0093
Zeaxanthin	12.258 $\pm$ 0.088	0.955 $\pm$ 0.021	0.1966 $\pm$ 0.0043
β -Apo-8'-carotenal	13.394 $\pm$ 0.141	0.939 $\pm$ 0.011	0.032 $\pm$ 0.0006
Canthaxanthin	13.977 $\pm$ 0.089	0.975 $\pm$ 0.008	0.1155 $\pm$ 0.0005
Astaxanthin	15.43 $\pm$ 0.066	0.965 $\pm$ 0.011	0.1034 $\pm$ 0.0009

¹⁾ K_m , Michaelis–Menten constant

²⁾ V_{max} , maximum reaction velocity

³⁾Conversions were calculated as a decrease in substrate content. Values represent an average of 3 independent incubations $\pm$ SD.

Influence of pH on enzyme activity The optimum pH for degradation activities of all substrates was 3.0 in this study (Fig. 2B), while the optimum pH value for carotenoid cleavage activities were reportedly 6.0 for *Pandanous amryllifolius* (27), 7.0 for *Sphingopyxis alaskensis* RB2256 and *Plesiocystis pacifica* SIR-1 (19), and 5.0 for *Pleurotus eryngii* (17). Use of the optimal pH value will allow improvements in wine flavor and enzyme performance in an adverse industrial environment.

Non-volatile carotenoid enzyme reaction products Although optimum temperature and pH values of the crude enzyme were 50–60°C and 3.0, non-volatile products were generally unstable and the products were degraded at low pH values, high temperatures, and long incubation times. Therefore, optimized reacted conditions for maximum yields of non-volatile products were studied. A reaction temperature of 30°C and pH of 5.0 were the best conditions to avoid loss of non-volatile products. Products were then subjected to HPLC for separation and detection. The non-volatile breakdown products

of carotenes, xanthophylls, and apocarotenals were separated and tentatively identified on the basis of UV-Vis spectra and Mw using HPLC-DAD and LC-MS (Fig. 1 and Table 2).

β -Carotene was observed on all the HPLC graphs (Fig. 1A, 1B, and 1C) and two additional peaks were observed on the HPLC graphs (Fig. 1B and 1C), indicating that the cleavage activity of the crude enzyme on β -carotene displayed a higher chemical conversion to products. Moreover, an additional peak with a retention time less than the β -carotene retention time was observed at pH 4.0 compared with the reaction at pH 5.0 (Fig. 1B and 1C). LC-MS analytical results for products 1–3 are shown in Fig. 1D, 1E, and 1F. Based on a measured m/z value of 553.4398 for the molecular ion $[M+H]^+$ and measured absorption maxima at 445 nm in an HPLC eluent (Fig. 1D), product 1 was identified as mutatochrome. The mass spectrum of product 2 also exhibited a mass peak at m/z =553.4398 with measured absorption maxima of 443 nm in an HPLC eluent (Fig. 1E), indicating that product 2 was β -carotene -5,6-epoxide. Product 3 was the substrate β -carotene with an expected molecular ion $[M+H]^+$ value of m/z =

Fig. 2. Effect of temperature (A) and pH (B) on the activity of crude enzyme degradation of carotenoid substrates.

535.4291 and absorption maxima at 450 nm in an HPLC eluent (Fig. 1F).

Based on LC-MS analysis of cleavage products (Table 2), epoxidation of substrates occurs as a first step for catalysis of the crude enzyme (Fig. 3). On the basis of identified cleavage products, the first step of β -carotene cleavage by the crude enzyme at pH 5.0 was in agreement with the cleavage sequence of other substrates. However, mutatochrome was obtained via 2 cleavage pathways of directly from β -carotene and by conversion from β -carotene-5,6-epoxide. Thus, the enzymatic

sequence at pH 4.0 may have 2 cleavage pathways (Fig. 3) and mutatochrome was the major non-volatile breakdown product.

The crude enzyme could cleave β -carotene to form an additional product at pH 4.0 that was not formed at pH 5.0, and the content of the new product was notably higher than the content of product 2 (Fig. 1C). This cleavage pattern was consistent with a previous report on the reaction sequence for formation of non-volatile and volatile compounds during heating of β -carotene (28).

Volatile enzyme reaction products of carotenoids β -Ionone and β -ionone-5,6-epoxide were the major reaction products isolated during incubation of β -apo-8'-carotenal and β -carotene, and concentrations of β -ionone and β -ionone-5,6-epoxide in reaction samples were higher than in controls (Table 3). Although the yield of β -cyclocitral was high under the reaction conditions used herein, β -cyclocitral was also detected in controls, indicating that β -cyclocitral can also be formed via auto-cleavage of substrates. Formation of β -ionone-5,6-epoxide may result from cleavage of β -carotene-5,6-epoxide. β -Carotene-5,8-epoxide is apparently a direct precursor of dihydroactinidiolide, so the yield of dihydroactinidiolide was significantly ($p < 0.05$) higher for degradation of β -carotene at pH 4.0, compared with reaction at pH 5.0. Similarly, 3-hydroxy- β -ionone, 3-oxe- β -ionone, and α,β -dihydro- β -ionone were released from zeaxanthin, canthaxanthin, and astaxanthin respectively (Table 3). 3-Hydroxy- β -ionone and 3-oxe- β -ionone have been described as flavor precursors in caramobola, nectarines, and tobacco (29). α,β -Dihydro- β -ionone is a major volatile of osmanthus flowers and strawberry fruits. It has been reported that α,β -dihydro- β -ionone is synthesized from α -ionone or β -ionone by catalytic hydrogenation. Thus, enzymatic cleavage of astaxanthin into α,β -dihydro- β -ionone is a major route for production of volatile compounds.

LC-MS and GC-MS analyses indicated that the crude enzyme from *S. pasteurii* TS-82 was able to catalyze substrates (30) and cleave substrates at 9-10 and 9'-10' double bonds (15,27), with β -ionone, β -ionone-5,6-epoxide, 3-hydroxy- β -ionone, 3-oxe- β -ionone and α,β -dihydro- β -ionone being the dominant volatile reaction products. No detection of C_{14} and C_{17} dialdehyde was probably because amounts were less than the analytical detection limit, or compound instability (31). Although the non-volatile carotenoid cleavage products listed above were detected at concentrations per million, all carotenoids

Table 2. Non-volatile Degradation Products of Carotenes Tentatively Identified using HPLC-DAD and HPLC-MS

Substrate	Product retention time (min)	[M+H] ⁺	Tentative identification
β -Carotene	28.667	553.4398	Mutatochrome
	30.837	553.4398	β -Carotene -5,6-epoxide
β -Apo-8'-carotenal	22.127	433.3101	Apo-8'-carotenal-5,6-epoxide
	23.785	409.3034	Apo-10'-zeaxanthinal-5,6-epoxide
Zeaxanthin	24.292	585.4279	Zeaxanthin epoxide
	24.302	407.2939	Apo-10'-canthaxanthinal-5,6-epoxide
Canthaxanthin	24.649	581.3977	Canthaxanthin epoxide
	19.882	423.4543	Apo-10'-astaxanthinal-5,6-epoxide
Astaxanthin	20.619	613.3915	Astaxanthin epoxide

Fig. 3. Reaction sequence for formation of non-volatile and volatile compounds during reaction of a β -carotene mixture with the enzyme from *S. pasteurii* TS-82 (30°C, pH 5.0 and 4.0).

were readily degraded under the experimental conditions used herein. Obtained yields of volatiles and the presence of only trace amounts of non-volatile degradation products suggested a gap in the mass balance. Further investigation is underway to fully elucidate the enzymatic reaction pathway. Compositional analysis of the crude enzyme is an important topic for a research framework, and

purification and identification of the crude enzyme is underway. Results presented in this study provide basic technical research information.

Techniques for determination of carotenoid breakdown products have also been investigated with respect to carotenoid cleavage enzymes from other microorganisms, such as *Marasmius scorodoni*

Table 3. Tentatively identified volatile degradation products of carotenes and xanthophylls

Substrate	Degradation product	Yield (reaction) (mg/L)	Yield (control) (mg/L)	GC-MS (<i>m/z</i>)
β -Carotene ¹⁾	β -Ionone ²⁾	0.44	tr	192(M ⁺), 177(100), 135, 107, 105
	β -Ionone-5,6-epoxide	0.15	tr	208(M ⁺), 135, 124, 123(100)
	Dihydroactinidiolide	0.06	tr	180(M ⁺), 137, 111(100), 110, 109
	β -Cyclocitral	0.23	0.11	152(M ⁺), 137, 123, 109, 67(100)
	2-Hydroxy-2,6,6-trimethyl cyclohexanone	tr ⁴⁾	ND ⁵⁾	156(M ⁺), 128, 110, 95, 71(100)
β -Apo-8'-carotenal ¹⁾	β -Ionone ²⁾	0.28	tr	192(M ⁺), 177(100), 135, 107, 105
	β -Ionone-5,6-epoxide	0.15	tr	208(M ⁺), 135, 124, 123(100)
	Dihydroactinidiolide	tr	tr	180(M ⁺), 137, 111(100), 110, 109
	β -Cyclocitral	0.24	0.10	152(M ⁺), 137, 123, 109, 67(100)
Zeaxanthin ³⁾	2-Hydroxy-2,6,6-trimethyl cyclohexanone	tr	ND	156(M ⁺), 128, 110, 95, 71(100)
Canthaxanthin ³⁾	3-Hydroxy- β -ionone	0.4	tr	208(M ⁺), 193(100), 175, 147, 131
Canthaxanthin ³⁾	3-Oxo- β -ionone	0.64	tr	206(M ⁺), 163(100), 121, 91, 43
Astaxanthin ³⁾	α , β -Dihydro- β -ionone	0.52	tr	194(M ⁺), 151, 136(100), 121, 91

¹⁾ZB-WAX, 60 m×0.25 mm i.d., 0.25 μ m.

²⁾Compound identified using a reference standard.

³⁾DB-5MS, 60 m×0.25 mm i.d., 0.25 μ m.

⁴⁾tr stands for trace amount (<0.05 mg/L).

⁵⁾ND stands for not detected.

and *Sphingopyxis alaskensis* RB2256 (17,19). Enzyme catalysis used to produce molecules for perfume, flavors, and fragrances has the advantage of allowing products to be labeled as natural (32). Therefore, natural flavors of β -ionone, β -ionone-5,6-epoxide, 3-hydroxy- β -ionone, 3-oxe- β -ionone, and α,β -dihydro- β -ionone from this study are promising as natural food ingredients. Moreover, it has been reported that β -ionone exerts anti-proliferative and cell-cycle regulatory effects in breast cancer cells, and suppresses mammary carcinogenesis (33). Lasting consumer interest in natural compounds reflects health and food quality concerns. In addition to production of bioflavors, the crude enzyme from *S. pasteurii* TS-82 can also be used as a tool in detergents and food bleaching applications.

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