JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2007,** *55,* 6705–6711 **6705**

Functional Characterization of Enone Oxidoreductases from Strawberry and Tomato Fruit

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Fragaria x ananassa enone oxidoreductase (FaEO), earlier putatively assigned as quinone oxidoreductase, is a ripening-induced, negatively auxin-regulated enzyme that catalyzes the formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF), the key flavor compound in strawberry fruit by the reduction of the α,β-unsaturated bond of the highly reactive precursor 4-hydroxy-5-methyl-2-methylene-3(*2H*)-furanone (HMMF). Here we show that recombinant FaEO does not reduce the double bond of straight-chain 2-alkenals or 2-alkenones but rather hydrogenates previously unknown HMMF derivatives substituted at the methylene functional group. The furanones were prepared from 4-hydroxy-5-methyl-3(2*H*)-furanone with a number of aldehydes and a ketone. The kinetic data for the newly synthesized aroma-active substrates and products are similar to the values obtained for an enone oxidoreductase from *Arabidopsis thaliana* catalyzing the α,β-hydrogenation of 2-alkenals. HMMF, the substrate of FaEO that is formed during strawberry fruit ripening, was also detected in tomato and pineapple fruit by HPLC-ESI-MS^n and became ^{13}C-labeled when D-[6-^{13}C]-glucose was applied to the fruits, which suggested that a similar HDMF biosynthetic pathway occurs in the different plant species. With a database search (http://ted.bti.cornell.edu/ and http://genet.imb.uq.edu.au/Pineapple/), we identified a tomato and pineapple expressed sequence tag that shows significant homology to FaEO. *Solanum lycopersicon* EO (SlEO) was cloned from cDNA, and the protein was expressed in *Escherichia coli* and purified. Biochemical studies confirmed the involvement of SlEO in the biosynthesis of HDMF in tomato fruit.

KEYWORDS: ***Fragaria x ananassa*; *Solanum lycopersicon*; enone oxidoreductase; 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; flavor formation**

INTRODUCTION

Among the volatiles that constitute strawberry (*Fragaria x ananassa*) flavor, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF, Furaneol) is the most important because of its high concentration (up to 55 mg/kg fruit fresh weight) and low odor threshold (10 ppb) (**Figure 1**) (*1*). HDMF has a distinctive sweet caramel-like flavor and was first isolated from pineapple (*Ananas comosus*) (*2*) and then from strawberry (*3*) and a number of different fruits (*1*). Radiotracer studies demonstrated the bioconversion of HDMF to its methyl ether, its β-D-glucopyranoside, and subsequently to the malonylated derivative of HDMF glucoside (*4*, *5*).

It has been postulated that compounds eliciting a caramel-like aroma have a planar enol-carbonyl substructure in a cyclic dicarbonyl compound (*6*). The heterocycles HDMF, 4-hydroxy-5-methyl-3(2*H*)-furanone (HMF, norfuraneol), and 5-(or 2)-ethyl-4-hydroxy-2(or 5)-methyl-3(2*H*)-furanone (EHMF, homofuraneol) all contain this structural element capable of forming a strong hydrogen bond and rank among the important flavor chemicals (**Figure 1**). These compounds are known to be products of the Maillard reaction between reducing sugars and amino acids and are common to a variety of thermally processed foods, but they have also been identified as natural constituents of fruits, insects, and fermented products, implying a biochemical formation pathway (*1*).

The first indication for the enzymatic formation of HDMF in strawberry fruit was provided by a study demonstrating the correlation between fruit-ripening stage and HDMF concentration (*7*). Incorporation experiments with radiolabeled precursors and substances labeled with stable isotopes revealed D-fructose-1,6-diphosphate as an efficient biogenetic precursor of HDMF (*4*, *8*). In strawberry, D-fructose-1,6-diphosphate is converted by an as yet unknown enzyme to 4-hydroxy-5-methyl-2-methylene-3(2*H*)-furanone (HMMF), which serves as substrate for an oxidoreductase recently isolated from ripe fruit (*9*).

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10.1021/jf071055o CCC: $37.00
Published on Web 07/18/2007

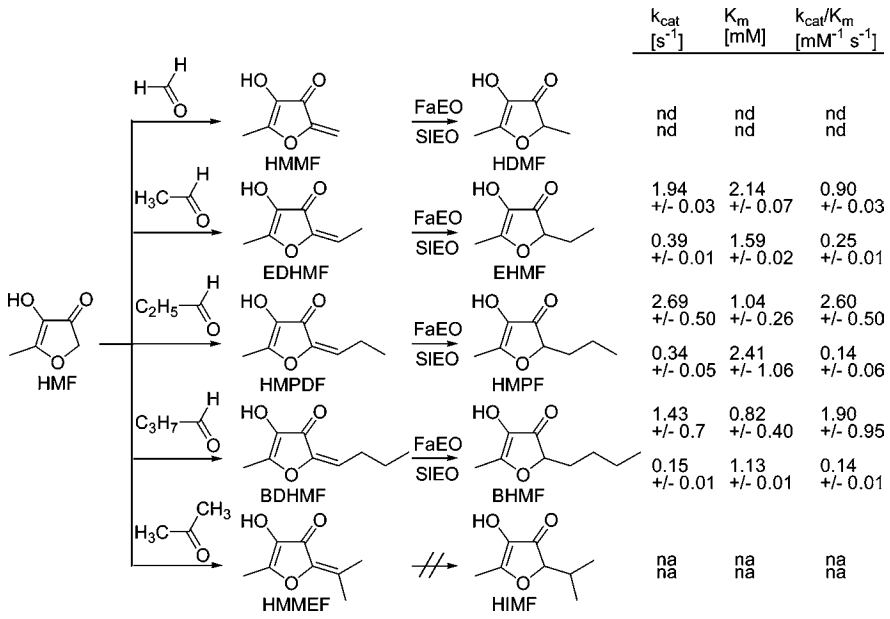

Figure 1. Chemical synthesis of substrates and kinetics of FaEO and SlEO. HMMF, EDHMF, HMPDF, BDHMF, and HMMEF starting from HMF and structures of the products HDMF, EHMF, HMPF, BHMF, and HIMF. Enzymatic assays were carried out with final concentrations of unsaturated hydroxyfuranones in the range of 0.14–5.7 mM in mixtures containing 1.5 μg of FaEO (or SlEO) and 12 mM NADH in 250 μL of phosphate buffer pH 7. The assays were run for exactly 35 min at 30 °C, and HPLC-ESI-MS^n was performed immediately. The amounts of formed HMPF and BHMF were calculated as "EHMF equivalents". K_m and V_{max} values were determined by fitting the data to the Hanes equation. All data points are averages of duplicates. nd = not determined, na = not applicable.

Sequence analysis of two peptide fragments showed identity with the protein sequence of a strongly ripening-induced, auxin-dependent putative quinone oxidoreductase (FaQR). Because the recombinant protein reduced only the artificial substrate 9,-10-phenanthrene quinone (K_m = 35 μM) out of a number of 1,2- and 1,4-quinones tested and formed HDMF from HMMF, the enzyme was renamed enone oxidoreductase (FaEO) (*9*).

In the present study, we report the synthesis and characterization of HMMF, the natural substrate of FaEO, and its derivatives as well as their biochemical conversion by FaEO. The identification of HMMF in HDMF-forming fruits led to the cloning, heterolog expression, and characterization of an enone oxidoreductase from *Solanum lycopersicon* (SlEO), which catalyzes the same reactions as FaEO.

MATERIALS AND METHODS

Materials. All reagents and solvents were obtained from Sigma-Aldrich, Taufkirchen, Germany, unless otherwise stated. D-[6-^{13}C]-glucose was obtained by Deutero GmbH, Kastellaun, Germany.

Plant Material. Strawberry fruits (*Fragaria x ananassa* Elsanta) were harvested at three different developmental stages (full sized green fruits, turning stage fruits, and full-ripe red fruits) from a commercial field. Cherry tomato (*Solanum lycopersicon cerasiforme*) plant material was harvested in different stages (buds, flowers, and fruits) from a private garden or was bought from a local market. Pineapple fruits (*Ananas comosus*) were obtained from a local supermarket.

RNA Isolation. Isolation of total RNA from different tissues of cherry tomatoes was performed with the RNeasy Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

cDNA Synthesis. The *SlEO* cDNA was synthesized using SuperScript III (Invitrogen, Karlsruhe, Germany) for RT-PCR and the specific primers 5′-GAC CAT GGG TAT GGA AGC TCT GTT ATC TTC-3′ containing the restriction site *Nco*I and 5′-GAC TCG AGA GGA ATG GGA TGA ATA ACA AC-3′ containing *Xho*I. PCR products were separated on a low melting agarose gel (1.5%). Bands of about 1200 bp were cut from the gel and purified by QIAquick Gel Extraction Kit (Qiagen). After ligation of the A-tailed insert into pGem T easy (Promega, Mannheim, Germany), the plasmid was transformed into JM109 *Escherichia coli* cells. The vector was purified and digested with endonucleases *Xho*I and *Nco*I, and after cutting off the phosphate groups, the insert was ligated into an *Xho*I and *Nco*I predigested expression vector pET29a(+) (Novagen, Calbiochem-Novabiochem GmbH, Schwalbach, Germany). The resulting recombinant plasmid was amplified in *E. coli* JM109 cells. Following sequencing they were transformed into BL21(DE3)plys *E. coli* host strain for expression.

Expression and Purification. The cloning and isolation of the full-length strawberry cDNA *FaEO* was conducted as described by Raab et al. (*9*). The expression and purification of the enzymes FaEO and SlEO were performed according to a published protocol (*9*).

Synthesis and Purification of HMMF and Its Derivatives. HMF (16.9 g, 43.8 mmol), copper(II)acetate (20 mmol), sodium acetate (25 mmol), and 12 mL of an aqueous formaldehyde solution (37%) were dissolved in 75 mL of acetic acid. For the synthesis of the derivatives, the formaldehyde solution was replaced by acetaldehyde (6.5 g, 148 mmol), propionaldehyde (7.1 g, 148 mmol), butyraldehyde (10.6 g, 148 mmol), or acetone (7.1 g, 148 mmol). The solution was heated to 50 °C and stirred for 1 h before being diluted with 150 mL of water. Products were isolated by diethyl ether (100 mL) extraction. The extract was dried over sodium sulfate and concentrated by distillation. The residue was again dissolved in 50 mL of water, extracted with 50 mL of diethyl ether, and concentrated to remove remaining acetic acid. Purification was performed by preparative silica gel thin layer chromatography (silica gel 60 F 254, 5 × 20 cm, 0.25 mm, Merck, Darmstadt, Germany) with pentane/diethyl ether 1:1 (v/v). The bands of interest were removed and extracted with diethyl ether. Products were finally purified by preparative RP18-HPLC-UV, extracted with diethyl ether, and analyzed by NMR.

Synthesis and Purification of the HMMF Thioether Adduct. HMMF (ca. 0.4 mmol) and 3-mercaptobenzoic acid (MBA) (0.39 mmol) were added to 10 mL of water, and the solution was stirred in the dark at room temperature overnight. The progress of the reaction was monitored by HPLC-ESI-MS^n, and the product was purified by preparative RP18 chromatography (16 × 4 cm, LiChrospher 100) using solvent mixtures consisting of water and acetonitrile (ACN) of decreasing polarity. The HMMF-MBA product eluted with 50% ACN. The fractions were combined and extracted with 50 mL of diethyl ether. The extract was dried over sodium sulfate, concentrated, and subjected to silica gel chromatography (25 × 2.5 cm, silica gel 60, 0.04−0.063

mm, Carl Roth GmbH, Karlsruhe, Germany) using mixtures of pentane and diethyl ether of increasing polarity. The product eluted with 100 mL of diethyl ether. The fractions were combined, concentrated, and analyzed by NMR.

Kinetic Data. Unsaturated hydroxy-3(2*H*)-furanones (0.14−5.7 mM final concentration) were added to an assay mixture containing 1.5 μg of purified enzyme, 12 mM NADH in 250 μL of Na_2HPO_4/NaH_2PO_4 buffer (0.1 M, pH 7) at 30 °C for 35 min. Products were quantified by HPLC-ESI-MS^n analysis. K_m and V_{max} values were determined by fitting the data to the Hanes equation using the regression program Hyper32 (version 1.00; http://homepage.ntlworld.com/john.easterby/hyper32.html). All data points were averages of duplicates.

Trapping of HMMF in Fruit. One to two milliliters of a 70% ethanolic solution containing 0.3 M MBA was injected into fruits on two consecutive days. Fruits injected with only a 70% ethanolic solution served as control.

Tracer Study. Twenty-one milligrams of D-[6-^{13}C]-glucose dissolved in 100 μL of water was injected into strawberry fruits in the turning ripening stage on two consecutive days. On the third day, 50 μL of a 0.6 M MBA solution was injected into the fruits. In total, fruits were stored for 3 days at room temperature before workup.

Isolation of the HMMF Thioether Adduct. Fruits were cut into small pieces and immediately frozen at −20 °C. Thawed fruits (10−20 g) were homogenized in an equal amount of water and centrifuged (10 min at 5000*g*), and the supernatant was applied onto a preconditioned XAD (adsorbent resin) column (30 cm × 1.5 cm). Polar compounds were removed with 100 mL of water, nonpolar substances were eluted with 100 mL of diethyl ether, and the semipolar metabolites were obtained by elution with 100 mL of methanol. The diethyl ether and methanol extracts were concentrated and analyzed by HPLC-ESI-MS^n.

Preparative RP18-HPLC-UV. A Knauer (Berlin, Germany) HPLC system equipped with a variable wavelength detector set at 280 nm and a preparative RP18 column (Synergi 4 μm Fusion, 250 × 15 mm, Phenomenex, Aschaffenburg, Germany) were used. Purifications were performed using a binary gradient starting from 100% A (H_2O with 0.1% HCOOH) and 0% B (ACN with 0.1% HCOOH) increasing to 20% B in 10 min and then to 100% B during the following 10 min at a flow rate of 5 mL/min.

HPLC-ESI-MS^n. HPLC-ESI-MS^n was performed using an Esquire 3000^{plus} system equipped with an ESI interface (Bruker Daltonics, Bremen, Germany) and an Agilent 110 HPLC system (Agilent, Waldbronn, Germany). The separation was carried out on a Luna RP18 column (100 A, 150 × 200 mm, 5 μm, Phenomenex). The LC gradient proceeded from 0% ACN and 100% water acidified with 0.05% HCOOH to 20% ACN and 80% acidic water in 10 min, in 10 min to 100% ACN, 2.5 min at these conditions, then back to 100% water and 0% ACN in 5 min at a flow rate of 0.2 mL/min. The detection wavelength was 280 nm. Mass spectra were acquired in the positive mode. The ESI nebulizer used nitrogen at a pressure of 30 psi, and nitrogen was used as dry gas with a flow of 9 L/min at a temperature of 330 °C. The scan range included the masses from 20 to 500 *m*/*z*, and the settings of the ion trap were optimized for each compound (smart parameter settings, Bruker Daltonics). Product ion scanning was performed with helium (3.56×10^{-6} bar) as collision gas and a collision energy of 1.0 V. Data acquisition and evaluation was conducted with Esquire 5.1 and DataAnalysis software (version 3.1) (Bruker Daltonics).

Nuclear Magnetic Resonance Spectroscopy. ^{1}H NMR spectra were recorded at 500.13 MHz with a Bruker DRX 500 spectrometer (Bruker, Karlsruhe, Germany). The compounds were dissolved in either deuterated chloroform, acetone, or methanol. The chemical shifts were referred to the solvent signal. The one-dimensional and two-dimensional COSY, HMQC, and HMBC spectra were acquired and processed with standard Bruker software (XWIN-NMR).

HRGC-MS. High-resolution gas chromatography mass spectrometry was performed with a Trace GC 2000 Ultra (Thermo Electron Corp., Dreieich, Germany) connected to a quadrupole (Trace DSQ) mass spectrometer and an autoinjector AI 3000. The GC was equipped with a split injector (1:20) and fitted with a DB-FFAP fused silica capillary column (30 m × 0.32 mm inner diameter; thickness of the film = 0.25 μm). The GC parameters were as follows: Initial temperature of 40 °C for 5 min, increased to 250 °C at 5 °C/min intervals. The helium gas flow rate was 3 mL/min. The EI-MS ionization voltage was 70 eV (electron impact ionization), and the ion source and interface temperatures were kept at 230 and 240 °C, respectively. The scan range was set from *m*/*z* 45 to 350. The data evaluation was performed using Xcalibur (version 1.4, Thermo Electron) software.

RESULTS

Synthesis of Substrates. Unsaturated hydroxyfuranones were synthesized by a Knoevenagel-type condensation of HMF with a number of aldehydes or a ketone (**Figure 1**) (*10*, *11*). One major problem was the difficulty in controlling the synthesis, which resulted in a high number of undesired reaction products. Necessary purification steps were hindered by the high instability of the target compounds (*11*). The α,β-unsaturated ketones are very reactive because alcohols, thiols, and water may add to the exocyclic double bond (*12*). The stability of the synthesis products increases with the length of the side chain. Therefore, HMMF is the most unstable substance but could be trapped with MBA as tautomeric thioether products for characterization. One- and two-dimensional NMR experiments (^{1}H-, COSY, HMQC, and HMBC) and comparison with the spectra of HDMF and EHMF confirmed the identity of the synthesized thioethers (**Tables S1a** and **S1b**, Supporting Information). Signals for all four possible tautomeric thioether forms were found in the NMR spectra, but because of their signal ratios of 3:2.5:1:1 and the low signal abundance only the carbons and hydrogens of the two major isomers (2′-{[(3-hydroxy-2-methyl-4-oxo-4,5-dihydrofuran-5-yl)methyl]-sulfanyl}benzoic acid (HMMF-MBA 1) and 2′-{[(4-hydroxy-2-methyl-3-oxo-2,3-dihydrofuran-5-yl)-methyl]-sufanyl}benzoic acid) (HMMF-MBA 2)) could be reliably assigned. Separation of the four isomers was not achieved. No coupling between the aromatic and the furanone part of the molecules was observed, but comparison of the signal area ratios enabled the assignment of the signals to definite molecule structures. Eight hydrogen signals were acquired for the major HMMF-MBA 1 isomer: four downfield shifted signals resulting from the aromatic system and four in a chemical shift range of 2.3−4.7 ppm representing the furanone moiety. The characteristic double-doublet signals at 3.35 and 3.65 ppm were tentatively assigned to the hydrogen atoms bound to C-6 in HMMF-MBA 1. The signals of the aromatic part of the second major HMMF-MBA isomer were almost identical to those of the first isomer, but the signals of the furanone moiety differed significantly. The singlet signal at 2.1 ppm was tentatively assigned to the hydrogen atoms bound to C-6. The proposed structure for HMMF-MBA 2 was further confirmed by the doublet signal at 5.0 ppm caused by the hydrogen atom bound to C-2 and the quartet signal for the methyl atoms appearing at 1.4 ppm in contrast to a singlet signal at 2.3 ppm as was observed for HMMF-MBA 1.

Because (2*E*)-2-ethylidene-4-hydroxy-5-methyl-3(*2H*)-furanone (EDHMF), (2*E*)-4-hydroxy-5-methyl-2-propylidene-3(2*H*)-furanone (HMPDF), (2*E*)-2-butylidene-4-hydroxy-5-methyl-3(2*H*)-furanone (BDHMF), and 4-hydroxy-5-methyl-2-(1-methylethylidene)-3(2*H*)-furanone (HMMEF) (**Figure 1**) are much more stable in a nonpolar milieu than HMMF and their syntheses yielded significantly more product than the HMMF synthesis, these hydroxyfuranones could be analyzed by NMR (^{1}H-, COSY, HMQC, and HMBC) without prior derivatization (**Tables S2a**−**S2c**, Supporting Information) (*11*). Tautomeric forms were not observed. The spectra of all molecules showed a singlet at 2.3 ppm for the CH_3-group at position C-1. Long-range ^{1}H, ^{13}C couplings were observed in the HMBC spectrum

between the methyl hydrogen atoms and the C-2 and C-3 carbon atoms of the dihydrofuranone ring system, respectively. By elongation of the carbon chain at the exocyclic double bond, the signals for the hydrogens and carbons of the CH_2 groups at positions C-7 and C-8 shifted high field compared with the signals of the CH_3 group. The structures of the side chains were also confirmed by ^1H, ^1H spin couplings of the neighboring hydrogen atoms at positions 6−9.

Enzymatic Conversion. For substrate screening, we used the synthesized compounds (HMMF, EDHMF, HMPDF, BDHMF, HMMEF) and diverse naturally occurring and non-natural α,β-unsaturated carbonyl compounds (2*E*-hexenal, 3*E*-hexen-2-one, 5-methyl-3*E*-hexen-2-one, cyclohexa-1,2-dione, buta-2,3-dione, coumarin, astaxanthin, cinnamon aldehyde, *p*-coumaric acid, ferulic acid, dimethoxycinnamic acid, benzil) together with recombinant FaEO, produced by FaEO expressing *E. coli* and NADH and analyzed the products by HRGC−MS and HPLC-ESI-MS^n. Control experiments were performed with protein fractions from *E. coli* cells transformed with the empty vector. Only the newly synthesized compounds HMMF, EDHMF, HMPDF, and BDHMF except HMMEF were converted to their saturated derivatives. The enzymatic products HDMF and EHMF were identified by their electrospray ionization and electron impact mass spectra in comparison with authentic reference material (**Figures S1** and **S2**, Supporting Information). The structures of HMPF and BHMF were tentatively assigned because of their fragmentation pattern (**Figures S1** and **S2**, Supporting Information).

To calculate the K_m and k_{cat} values, we used EDHMF, HMPDF, and BDHMF in concentrations ranging from 0.14 to 5.7 mM with an excess of NADH (12 mM). HMMF was too unstable to obtain reliable values. The amounts of the product EHMF were determined by HPLC-ESI-MS^n with the help of a calibration curve. The lack of reference material for HMPF and BHMF forced us to use the EHMF standard curve to calculate the amounts of HMPF and BHMF as "EHMF equivalents", which is justified because of the similar structure and ionization behavior. Overall, the values fell in the same order of magnitude (**Figure 1**). HMPDF exhibited the highest k_{cat} value (2.69 s^{-1}) and catalytic efficiency (k_{cat}/K_m of 2.60 mM^{-1} s^{-1}), but the K_m values decreased with decreasing side chain lengths (2.14, 1.04, and 0.82 mM).

In Vivo Studies for the Detection of HMMF-MBA. Although HDMF has been detected in a number of fruits, its biosynthetic pathway has only been investigated in strawberries until now. Because HMMF had already been identified as precursor of HDMF in strawberry fruit after derivatization with MBA (*9*), HDMF forming fruits such as tomato, raspberry, pineapple, kiwi, and mango were treated with an ethanolic solution of the trapping reagent (*4*, *7*). We wanted to know whether HDMF is formed in the different fruits by the same pathway as already determined in strawberry. Apples that do not produce HDMF as well as fruits injected with ethanol served as controls. The HMMF-MBA thioether adduct was identified by its retention time (19.1 min) and MS^2 data showing characteristic fragments at *m*/*z* 281, 263, 155, and 127 (**Figure 2A**). The adduct was clearly detected by HPLC-ESI-MS^n in tomatoes and pineapples (**Figure 2B**,**C**). We found traces of the thioether in raspberry but were unable to carry out MS^n experiments because of the limited amount. The HMMF-MBA adduct was not detected in kiwi and mango. The levels of the thioether mirrored the levels of HDMF because GC−MS analysis showed that tomato and pineapple contained appreciable amounts of HDMF (>0.1 mg/kg fresh weight), whereas only

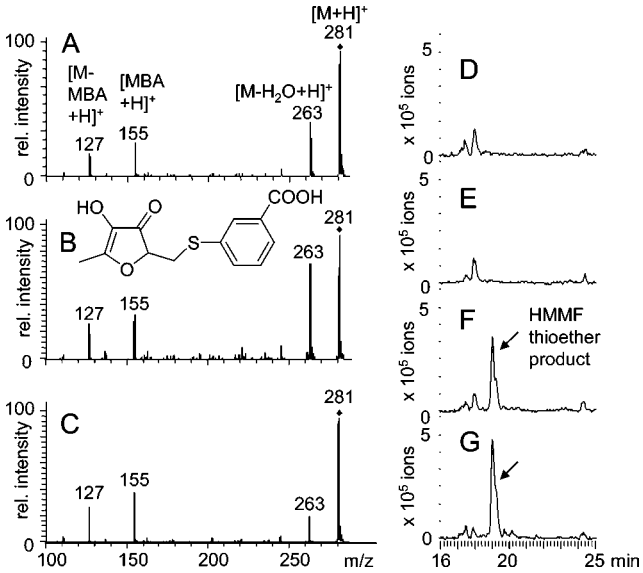

Figure 2. HPLC-ESI-MS^n analysis of the HMMF thioether product. Product ion spectrum of *m*/*z* (+) 281 of the synthesized reference compound (**A**), of the compound isolated from pineapple after treatment with MBA (**B**), and isolated from tomato treated with MBA (**C**). Extracted ion chromatogram *m*/*z* (+) 281 of extracts obtained from control fruits (**D**) and strawberries after the injection of MBA into fruits of the green (**E**), pink (**F**), and red (**G**) ripening stage. Fruits were treated for 2 days with 0.6 M ethanolic MBA solution. After freezing and thawing, the fruits were processed by a XAD solid-phase column, and the obtained diethyl ether fraction was analyzed by HPLC-ESI-MS^n under the same conditions.

traces were detected in raspberry and the concentrations in kiwi and mango were lower than our detection limit of about 0.01 mg/kg fresh weight. The controls were devoid of the thioether product (**Figure 2D**). Levels of HMMF correlated with the ripening stage of the fruits because the thioether product was only found in strawberry in the pink and red ripening stage but not in green immature fruits when determined under the same conditions (**Figure 2E**−**G**).

In Vivo Studies for the Detection of ^{13}C-HMMF-MBA. It had already been shown that HDMF becomes ^{13}C- and ^2H-labeled when isotopically labeled glucose is injected into ripening strawberry fruits (*4*, *8*). Thus, to confirm HMMF as the natural precursor of HDMF, we applied 100 μL of an aqueous solution of 21 mg of D-[6-^{13}C]-glucose into detached pink strawberry fruits. After 2 days, 50 μL of a 0.6 M ethanolic solution of MBA was injected. HPLC-ESI-MS^n analysis of extracts showed that the labeling degree of the thioether caused by the natural abundance of ^{13}C (determined by the M + 1 signal) increased from 13% in the untreated control fruits, up to 33% in fruits treated with D-[6-^{13}C]-glucose (**Figure 3A**,**D**). The MS^n experiments confirmed that the ^{13}C was exclusively integrated into the hydroxyfuranone moiety (**Figure 3B**).

Homologous Enzyme from Tomato. The demonstration of HMMF as natural precursor of HDMF in fruit of different species let us suppose that these fruits contain enzymes similar to FaEO. We found a putative quinone oxidoreductase sequence similar to FaEO in the tomato EST database (http://ted.bti.cornell.edu/) and pineapple database (http://genet.imb.uq.edu.au/Pineapple/) (**Figure 4**). The gene from tomato was cloned and expressed in *E. coli*. It shows 63 and 71% identity with *Fa*EO on the nucleotide and amino acid levels, respectively (**Table S3**, Supporting Information). It is also predicted to contain a chloroplast transit peptide at the N-terminus unlike FaEO (*13*). The recombinant enzyme lacking the signal peptide accepted

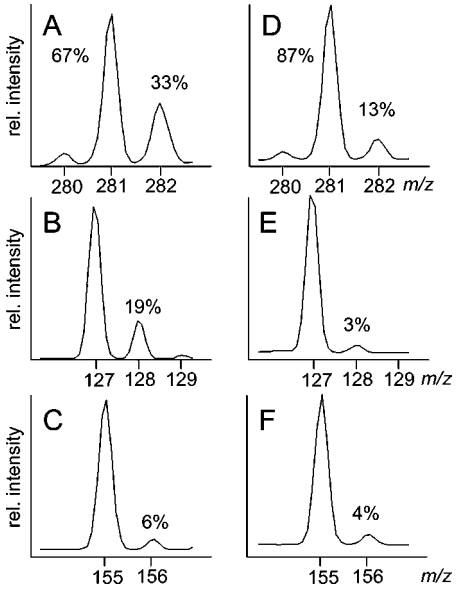

Figure 3. HPLC-ESI-MS^n analysis of the HMMF thioether product isolated from strawberry fruits treated with MBA. Sections of the product ion mass spectra showing the pseudo molecular ion $[M + H]^+$ at *m*/*z* 281 (**A**, **D**), the $[M\text{-MBA} + H]^+$ ion at *m*/*z* 127 representing the hydroxyfuranone moiety in the molecule (**B**, **E**), and the $[MBA + H]^+$ ion (**C**, **F**). Strawberries were treated with D-[6-^{13}C]-glucose and MBA (**A**, **B**, **C**) or only with MBA (control) (**D**, **E**, **F**). After freezing and thawing, the fruits were processed by a XAD solid-phase column, and the obtained diethyl ether fraction was analyzed by HPLC-ESI-MS^n.

HMMF, EDHMF, HMPDF, and BDHMF as substrates and reduced them to their saturated derivatives HDMF, EHMF, HMPF, and BHMF, respectively (**Figures 1** and **S3**, Supporting Information). HMMEF and straight-chain substrates were not accepted as observed for FaEO. SlEO showed k_{cat} values (0.15−0.39 s^{-1}) generally lower than those of FaEO (0.82−2.14 s^{-1}), by a factor of 5−10, but similar K_m values (1.13−2.41 mM, respectively, 0.82−2.14 mM). Overall, the substrates were less efficiently reduced by the tomato enzyme with a slight preference for EDHMF. We designate this protein as SlEO (*Solanum lycopersicon* enone oxidoreductase) because of the catalyzed reaction.

DISCUSSION

Until now, only little was known about the physiological roles of NAD(P)H-dependent quinone oxidoreductases in plants. During a study to uncover genes that perform antioxidant functions, a gene encoding an NADPH/quinone oxidoreductase-like protein was isolated from *Arabidopsis thaliana* (*14*) (**Figure 4**). The recombinant protein expressed in *E. coli* showed an NADPH-dependent oxidoreductase activity to reduce quinones (*15*). Upon a search for more physiologically relevant substrates, cytotoxic 2-alkenals and oxenes were identified as potential in vivo substrates (*16*). The α,*β*-unsaturated bond of 2-alkenals was specifically reduced, but the aldehyde group was retained. On the basis of this unique activity, the name alkenal reductase (AER) has been recommended. Only two proteins have been reported to have AER activity, AER from *Arabidopsis* (AtAER) and leukotriene B4 12-hydroxy dehydrogenase from rat (*17*). The AER-catalyzed reaction significantly lowers the toxicity of reactive carbonyls because the electrophilicity of the α-carbon, which reacts with target molecules, is diminished by the reduction of the unsaturated bond.

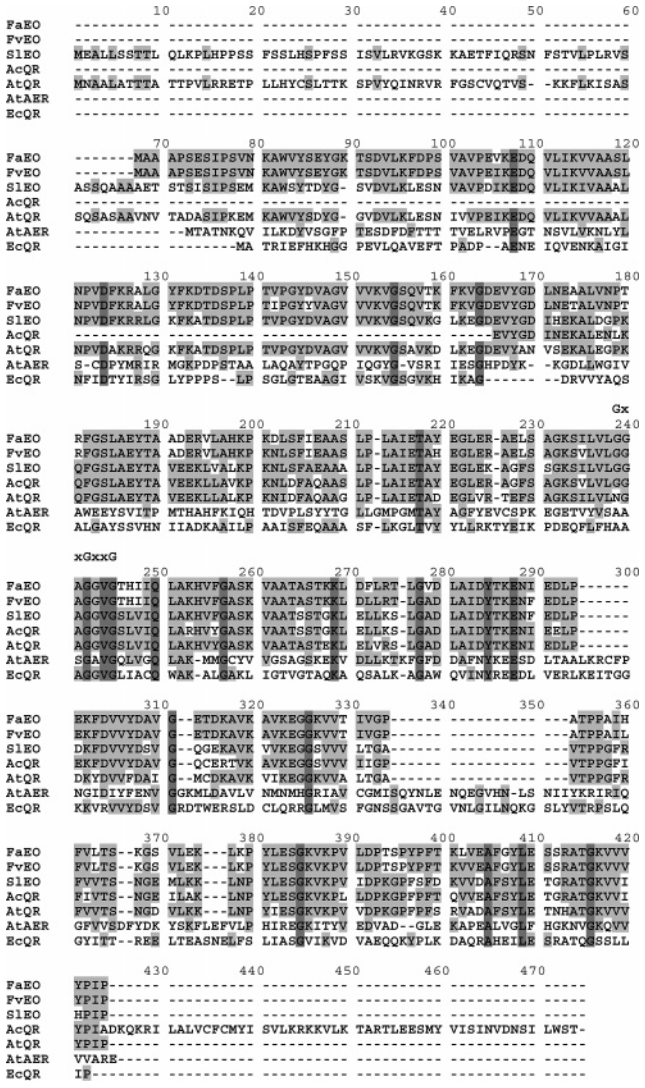

Figure 4. Amino acid sequence comparison of the strawberry FaEO protein and putative quinone oxidoreductases from higher plants and *E. coli*. The sequences were aligned using the ClustalW program. Consensus amino acids are shaded. The NAD(P)H binding motif G(A)XXGXXG is marked. The accession numbers are AY048861 for *Fragaria x ananassa* (FaEO), AJ001445 for *Fragaria vesca* (FvEO), TC124720 for *Solanum lycopersicon* (SlEO), JBW022G03 for *Ananasa comosa* (AcQR), AF451799 for *Arabidopsis thaliana* (AtQR), Q39172 for *Arabidopsis thaliana* (AtAER), and AAC43145 for *Escherichia coli* (EcQR).

In this study, we show that FaEO reduces the double bond adjacent to the carbonyl group in HMMF, EDHMF, HMPDF, and BDHMF similar to AER although FaEO was putatively assigned earlier as quinone oxidoreductase (**Figure 1**). HMMEF was not accepted probably because of the second methyl group, which causes steric hindrance. Accurate kinetic data were obtained for all substrates except HMMF, which was too unstable in aqueous solution. However, the conversion of HMMF to HDMF was clearly demonstrated by HPLC-ESI-MS^n analysis (*9*). Because the K_m and k_{cat} values for the furanone substrates fell in the same range, we assume similar data for HMMF (**Figure 1**). The specificity (k_{cat}/K_m) ranged from 1.4 to 2.7 mM^{-1} s^{-1}. These values differ significantly from the values obtained with 9,10-phenanthrene quinone (PQ) (K_m = 12 μM, k_{cat}/K_m = 650 mM^{-1} s^{-1}), a synthetic substrate that is accepted by FaEO and also by AtAER (K_m = 0.65 μM, k_{cat}/K_m = 151 mM^{-1} s^{-1}) (*9*, *15*). However, in contrast to FaEO, the

alkenal reductase from *A. thaliana* catalyzes the reduction of a number of 1,4-quinones (*15*). A recently crystallized *E. coli* quinone oxidoreductase (EcQR) also catalyzes the NAD(P)H-dependent reduction of quinone substrates but shows only 25 and 25% identity with FaEO and AtAER (*18*) (**Figure 4** and **Table S3**, Supporting Information).

EHMF, the product formed by FaEO from EDHMF, is an important constituent of soy sauce and cheese and determines their aromas. In soy sauce, it is assumed that EHMF is formed by the yeasts from an unknown precursor that is produced by the amino-carbonyl reaction of a pentose with an amino acid during heating (*19*). As there are NAD(P)H-dependent oxidoreductases known in yeasts, it is possible that EDHMF plays a role in the biosynthesis of EHMF during soy sauce preparation (*20*). The substrates HMPDF and BDHMF as well as the resulting enzymatic products HMPF and BHMF have not yet been described.

Recently, the presence of HMMF in ripening strawberry fruits was demonstrated by trapping the unstable α,*β*-unsaturated carbonyl compound with MBA as thioether adduct (*9*). Here, we prove that HMMF is also produced by tomato, pineapple, and raspberry fruits that contain appreciable amounts (>0.1 mg/kg) of HDMF. Because only minor levels of HDMF have been found in mangoes and kiwis, it is possible that we could not detect HMMF in these fruits. The highest levels of HMMF were detected in fully ripe, red fruits consistent with the ripening-induced expression of FaEO and the accumulation of HDMF during the late stages of strawberry ripening (**Figure 2**). Feeding experiments with D-[6-^{13}C]-glucose in strawberry fruits have already demonstrated the incorporation of the labeled carbon into HDMF (*8*). In line with the assumption that HMMF is the precursor of HDMF, our results show that also HMMF becomes ^{13}C-labeled when D-[6-^{13}C]-glucose is injected into ripening strawberry fruits. Together, these data suggest that HDMF is synthesized in the different fruit species by a similar pathway.

Therefore, we screened cDNA databases for sequences similar to FaEO and found a full-length cDNA in the tomato expression library (http://ted.bti.cornell.edu/) and a partial sequence in the pineapple cDNA database (http://genet.imb.uq.edu.au/Pineapple/). FaEO shows 71 and 73% identity with the protein sequence from tomato (SlEO) and pineapple (AcQR), respectively (**Figure 4** and **Table S3**, Supporting Information). SlEO carries a putative transit peptide of about 60 amino acids at the N-terminus that probably directs the protein into chloroplasts (**Figure 4**). A signal peptide has not been found for FaQR. But since D-fructose-1,6-diphosphate, the starting material for HDMF biosynthesis, is present in chloroplasts and the cytosol, it is conceivable that HDMF formation proceeds in both compartments. We were able to clone SlEO and functionally express the protein in *E. coli*. The purified enzyme reduced HMMF, EDHMF, HMPDF, and BDHMF with slightly lesser efficiency than FaEO (**Figure 1**). Thus, it seems that SlEO is involved in the biosynthesis of HDMF in tomato fruit. A putative QR protein sequence from *A. thaliana* (AtQR) was also found by BLAST search showing 69 and 70% identity with FaEO and SlEO, respectively, although AtAER displays only 27 and 28% identity with these proteins. The function of AtQR remains to be clarified.

The major in vivo function of the enone oxidoreductases from strawberry and tomato is the conversion of HMMF to HDMF as demonstrated by the activity-guided isolation of FaEO (*9*), the conversion of HMMF by recombinant FaEO and SlEO, the detection of HMMF in ripening strawberry and tomato fruit, and the conversion of labeled glucose to HMMF and finally to HDMF. EDHMF, HMPDF, and BDHMF have not been found in nature up to now. Although HMMF is very reactive and unstable, it is the precursor of HDMF in fruits. This implies that the supposed, but not yet characterized, enzyme that forms HMMF in vivo must either strongly protect its product or immediately transfer it to the detoxifying EOs after its production. Otherwise, HMMF could cause severe damage as it might react with essential components of the cell. Therefore, FaEO and SlEO probably perform, similarly to AtAER, a detoxification reaction (*15*, *16*).

This article demonstrates that not only strawberry but also tomato, and likely other fruit such as pineapple, express an enzyme that converts HMMF into HDMF, which is an important flavor compound. It also shows that the substrate specificity of both tomato and strawberry enzymes is toward hydroxyfuranones. Thus, the data provide the foundation for the biotechnological improvement of the aroma composition of agronomically relevant fruits.

ABBREVIATIONS USED

*At*AER, *Arabidopsis thaliana* alkenal reductase; BDHMF, (2*E*)-2-butylidene-4-hydroxy-5-methyl-3(2*H*)-furanone; BHMF, 2-butyl-4-hydroxy-5-methyl-3(2*H*)-furanone; EDHMF, (2*E*)-2-ethylidene-4-hydroxy-5-methyl-3(2*H*)-furanone; EHMF, 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone; FaEO, *Fragaria x ananassa* enone oxidoreductase; FaQR, *Fragaria x ananassa* quinone oxidoreductase; HDMF, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; HIMF, 4-hydroxy-2-isopropyl-5-methyl-3(2*H*)-furanone; HMF, 4-hydroxy-5-methyl-3(2*H*)-furanone; HMMEF, 4-hydroxy-5-methyl-2-(1-methylethylidene)-3(2*H*)-furanone; HMMF, 4-hydroxy-5-methyl-2-methylene-3(2*H*)-furanone; HMPDF, (2*E*)-4-hydroxy-5-methyl-2-propylidene-3(2*H*)-furanone; HMPF, 4-hydroxy-5-methyl-2-propyl-3(2*H*)-furanone; HRGC−MS, high-resolution gas chromatography mass spectrometry; SlEO, *Solanum lycopersicon* enone oxidoreductase; MBA, 3-mercaptobenzoic acid; NMR, nuclear magnetic resonance spectroscopy; XAD, adsorbent resin; HPLC-ESI-MS^n, high-performance liquid chromatography electrospray ionization multidimensional mass spectrometry.

ACKNOWLEDGMENT

We thank Yu Fan Yang and Regina Dick for technical support and Heather Coiner and Mark Somoza for correcting the manuscript.

Supporting Information Available: **Table S1a**: NMR signal assignments of HMMF-MBA 1. **Table S1b**: NMR signal assignments of HMMF-MBA 2. **Table S2a**: ^{1}H NMR signal assignments of BDHMF. **Table S2b**: ^{1}H NMR signal assignments of HMPDF. **Table S2c**: ^{1}H NMR signal assignments of EDHMF. **Table S3**: Amino acid identities calculated by ClustalW of protein sequences for proven and putative enone and quinone oxidoreductases. **Figure S1**: HPLC-ESI-MS^n analysis of FaEO substrates and corresponding products. **Figure S2**: GC−MS analysis of FaEO substrates and corresponding products. **Figure S3**: SDS-PAGE analysis of purified recombinant FaEO and SlEO. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review April 11, 2007. Revised manuscript received May, 31, 2007. Accepted May 31, 2007. This work was supported by Degussa AG.

JF071055O