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Research paper

Biochemical characterization and FAD-binding analysis of oleate hydratase from *Macrococcus caseolyticus*

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ABSTRACT

A putative fatty acid hydratase gene from *Macrococcus caseolyticus* was cloned and expressed in *Escherichia coli*. The recombinant enzyme was a 68 kDa dimer with a molecular mass of 136 kDa. The enzymatic products formed from fatty acid substrates by the putative enzyme were isolated with high purity (>99%) by solvent fractional crystallization at low temperature. After the identification by GC–MS, the purified hydroxy fatty acids were used as standards to quantitatively determine specific activities and kinetic parameters for fatty acids as substrates. Among the fatty acids evaluated, specific activity and catalytic efficiency (k_{cat}/K_m) were highest for oleic acid, indicating that the putative fatty acid hydratase was an oleate hydratase. Hydration occurred only for *cis*-9-double and *cis*-12-double bonds of unsaturated fatty acids without any *trans*-configurations. The maximum activity for oleate hydration was observed at pH 6.5 and 25 °C with 2% (v/v) ethanol and 0.2 mM FAD. Without FAD, all catalytic activity was abolished. Thus, the oleate hydratase is an FAD-dependent enzyme. The residues G29, G31, S34, E50, and E56, which are conserved in the FAD-binding motif of fatty acid hydratases ($GXGXXG_{(A/S)}X_{(15-21)}E_{(D)}$), were selected by alignment, and the spectral properties and kinetic parameters of their alanine-substituted variants were analyzed. Among the five variants, G29A, G31A, and E56A showed no interaction with FAD and exhibited no activity. These results indicate that G29, G31, and E56 are essential for FAD-binding.

1. Introduction

A hydroxy fatty acid is composed of hydroxyl group(s) and a long unbranched carbon chain with a carboxyl group at one end. Hydroxy fatty acids are derived from a variety of natural sources, including microorganisms, plant seed oils, plant waxes, plant cutin, tall oil, and cork [1]. They are useful chemical intermediates for synthesizing fine chemicals and pharmaceuticals. Some may protect plants against microbial infection, although the mechanisms of these anti-microbial effects are poorly understood [2,3].

The enzymes involved in the hydroxylation of fatty acids have been reported as P450 monooxygenase, hydroxylase, lipoxygenase, and hydratase. P450 monooxygenases from *Bacillus licheniformis*, *B. subtilis*, and *Candida tropicalis* catalyze the hydroxylation of several saturated fatty acids into omega-hydroxy fatty acids [4,5]. Oleate hydroxylase from the plant *Ricinus communis* [6], lipoxygenase from *Pseudomonas* sp. 42A2L [7], and fatty acid hydratases [8–10] convert oleic acid into 12-hydroxyoctadecanoic acid, (*E*)-10-hydroperoxy-8-octadecenoic acid, and 10-hydroxyoctadecanoic acid, respectively.

Fatty acid hydratase activity that converts oleic acid into 10-hydroxyoctadecanoic acid, was first reported in a myosin cross-reactive antigen (MCRA) protein from *Elizabethkingia meningoseptica* [10]. The MCRA protein from *Streptococcus pyogenes* is a flavoenzyme that catalyzes the hydroxylation of double bonds in unsaturated fatty acids [8]. Recently, a MCRA protein from *Bifidobacterium breve* has been identified as an FAD-dependent fatty acid hydratase that catalyzes not only hydration but also isomerization of linoleic acid to conjugated linoleic acid [9]. However, quantitative analyses and characterization of fatty acid hydratases have not been attempted because the products of the enzyme reaction are not commercially available. Therefore, these fatty acid hydratases have not been fully defined. Moreover, the FAD-binding residues in these fatty acid hydratases have not been determined and their roles are not fully understood.

In the present study, the hydroxylated products formed from fatty acid substrates by the putative fatty acid hydratase from

Abbreviations: FAD, flavin adenine dinucleotide; MCRA, myosin cross-reactive antigen; GC–MS, gas chromatography-mass spectrometry; LB, Luria-Bertani; IPTG, isopropyl-β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; PIPES, piperazin-*N*,*N*′-ethanesulfonic acid; MES, 4-morpholine ethanesulfonic acid; GR, glutathione reductase.

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Macrococcus caseolyticus were purified. The purified products were then used as standards in subsequent analyses. The biochemical properties of the enzyme were characterized quantitatively using manufactured standards, and the enzyme was exactly identified based on its substrate specificity. In addition, the FAD-binding properties of conserved residues in the FAD-binding motif were characterized.

2. Materials and methods

2.1. Materials

The expression vector pET-28a(+) was purchased from Novagen (San Diego, CA, USA). The expression host, *Esherichia coli* ER2566, and all restriction enzymes were purchased from New England Biolabs (Hertfordshire, UK). LB medium was purchased from BD Biosciences (San Jose, CA, USA). The substrate fatty acids and the cofactors FMN, NAD^+ and $NADP^+$ were purchased from Sigma (St. Louis, MO, USA). FAD was purchased from Tokyo Chemical Industry (Tokyo, Japan). The pre-stained maker proteins for the SDS-PAGE and gel filtration calibration kit were purchased from MBI Fermentas (Hanover, MD, USA) and GE Healthcare (Piscataway, NJ, USA), respectively.

2.2. Bacterial strains, plasmid, and culture conditions

M. caseolyticus KCTC 3582, *E. coli* ER2566, and plasmid pET-28a (+) were used as sources of genomic DNA encoding a putative fatty acid hydratase, host cells, and expression vector, respectively. *M. caseolyticus* was cultivated on 50 ml of growth medium containing 0.5% glucose, 0.5% yeast extract, 1.0% casein peptone, and 0.5% sodium chloride in a 250 ml flask at 37 °C with shaking at 200 rpm. The recombinant *E. coli* cells for protein expression were cultivated in LB medium (1.0% tryptone, 0.5% yeast extract, and 1.0% sodium chloride) in a 2000 ml flask containing 20 μg/ml kanamycin at 37 °C with shaking at 200 rpm. When the optical density of bacteria reached 0.6 at 600 nm, IPTG was added to the culture medium at a final concentration of 0.1 mM. The culture broth was then incubated with shaking at 150 rpm at 16 °C for 16 h to express the enzyme.

2.3. Gene cloning and site-directed mutagenesis

The gene encoding a putative fatty acid hydratase was amplified by PCR using *M. caseolyticus* genomic DNA as the template. The sequence of primers used for gene cloning was based on the DNA sequence of the putative fatty acid hydratase (MCRA) from *M. caseolyticus* JCSC5402 (GenBank accession number NC_011999.1). Forward (5′-GGGGCTAGCATG-TACTATAGTAATGGA-3′) and reverse primers (5′-AGGCTCGAGT-TATATCAAATTTGCTTC-3′) were designed to introduce the underlined *Nhe*I and *Xho*I restriction sites and were synthesized by Bioneer (Daejon, Korea). The amplified DNA fragment obtained by PCR with *pfu* polymerase (Solgent, Daejon, Korea) was extracted using the PCR purification kit (Promega, Fitchburg, WI, USA) and was ligated into the *Nhe*I and *Xho*I sites of pET-28a(+). The resulting plasmid was used to transform *E. coli* ER2566 as an expression host. Expression of the gene encoding the putative fatty acid hydratase was determined by SDS-PAGE. Mutations of the five conserved FAD-binding residues in the putative fatty acid hydratase were generated by site-directed mutagenesis using a QuikChange kit and protocol (Stratagene, Beverly, MA, USA). DNA sequencing was performed at the Macrogen facility (Seoul, Korea).

2.4. Enzyme purification

Cells were harvested from the culture broth by centrifugation at 6000 × *g* for 30 min at 4 °C, washed twice with 0.85% NaCl, and then resuspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM KCl and 10 mM imidazole. The resuspended cells were disrupted using Sonic Dismembrator (Fisher Scientific Model 100, Pittsburg, PA, USA) at 18 W on ice for 2 min. The unbroken cells and cell debris were removed by centrifugation at 13,000 × *g* for 20 min at 4 °C and the supernatant was filtered through a 0.45 μm filter. The filtrate was applied to an immobilized metal ion affinity chromatography cartridge (Bio-Rad, Hercules, CA, USA) equilibrated with 50 mM phosphate buffer (pH 8.0). The cartridge was washed extensively with the same buffer and the bound protein was eluted with a linear gradient of 10–500 mM imidazole at a flow rate of 1 ml/min. The eluate was collected and loaded immediately onto a Bio-Gel P-6 desalting cartridge (Bio-Rad) that had been previously equilibrated with 50 mM PIPES buffer (pH 6.5). The loaded protein was eluted with 50 mM PIPES buffer (pH 6.5) at a flow rate of 1 ml/min to obtain a solution of purified enzyme. All purification steps using the cartridges were carried out in a cold room at 4 °C with a Profinia protein purification system (Bio-Rad).

The protein expressed from plasmid pET-28a(+) contained thrombin restriction site (LeuValProArg/GlySer) at the N terminus between hexa-histidine tag and putative fatty hydratase. After the histidine tag in the expressed protein was hydrolyzed by treating a thrombin kit (Novagen), the added thrombin (35 kDa) was removed by untrafiltration using a Centricon (50 kDa cutoff, Millipore, Billerica, MA, USA). The resulting enzyme solution was applied to a His-bind column (Novagen) and the waste was obtained as a non-tagged enzyme. The column was washed with 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and the bound protein was eluted with 50 mM phosphate buffer (pH 8.0) containing 87.5 mM imidazole. The eluate was used as a histidine-tagged enzyme.

2.5. Determination of molecular mass

The subunit molecular mass of the putative fatty hydratase from *M. caseolyticus* was examined by SDS-PAGE under denaturing conditions, using the pre-stained maker proteins as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of the native enzyme was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 column (GE Healthcare). The purified enzyme solution was applied to the column and eluted with 50 mM PIPES buffer (pH 6.5) containing 150 mM NaCl at a flow rate of 0.3 ml/min. The column was calibrated with appoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa) as reference proteins (GE Healthcare), and the mass of the native enzyme was calculated by comparing with the migration length of reference proteins.

2.6. Enzyme assay

Fatty acid substrates were dispersed to a concentration of 4 mM in 4% (v/v) ethanol and the solution was homogenized at 10,000 rpm for 10 s using a homogenizer (IKA, Kuala Lumpur, Malaysia). The substrate and enzyme solutions were mixed in 1:1 (v/v) ratio. Unless otherwise stated, the enzymatic reaction was performed at 25 °C in 50 mM PIPES buffer (pH 6.5) containing 2 mM fatty acid, 2% (v/v) ethanol, 33 unit/ml (0.01 mg/ml) of enzyme, and 0.2 mM FAD for 10 min. To stop the reaction, the solution was acidified to pH 2.0 by adding 6 M HCl. One unit of the putative fatty hydratase activity was defined as the amount of enzyme required to

produce 1 nmol of 10-hydroxyoctadecanoic acid per min at 25 °C and pH 6.5. To measure specific activity, the enzymatic reactions were performed in 50 mM PIPES buffer (pH 6.5) containing 2 mM fatty acid at 25 °C for 10 min by adjusting the enzyme concentration from 33 to 3300 unit/ml.

2.7. Preparation of hydroxy fatty acid standards

The hydroxy fatty acid standards were prepared by solvent fractional crystallization at low temperature [11]. The enzymatic reaction was performed at 25 °C in 50 mM PIPES buffer (pH 6.5) containing 10 mM fatty acid, 2% (v/v) ethanol, and 3300 unit/ml of enzyme for 16 h. The reaction products thus obtained were extracted with an equal volume of ethyl acetate. The solvent was removed from the extract using a rotary evaporator. A mixture of 30% acetonitrile and 70% acetone was then added to the extracted fatty acid solution at room temperature. The solution was cooled in an ultra low temperature freezer for 24 h at −80 °C. The liquid, which comprised the unsaturated fatty acid fraction, was removed at room temperature. The solvent was removed from the solid extract using a rotary evaporator to obtain the hydroxy fatty acid fraction. This fractionization procedure was repeated three times. The hydroxy fatty acid products were obtained with high purity (>99%) and were used as standard compounds in subsequent analyses.

2.8. Effects of reaction conditions on enzyme activity

The putative fatty hydratase was obtained after incubation at 25 °C with 10 mM EDTA for 1 h followed by overnight dialysis at 4 °C against 50 mM PIPES buffer (pH 6.5). The effect of metal ions on enzyme activity was investigated using the EDTA-treated enzyme in the presence of 1 mM Mg^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Ba^{2+}, Zn^{2+}, Fe^{2+}, or Cu^{2+}. To examine the effect of pH on enzyme activity, the pH was varied from 5.5 to 7.5 using 50 mM MES buffer (pH 5.5–6.5) and 50 mM PIPES buffer (pH 6.5–7.5). The effect of temperature on enzyme activity was investigated by varying the temperature from 15 to 35 °C. The effect of temperature on enzyme stability was evaluated over the same temperature range for 120 min. Samples were withdrawn at specific time intervals and the residual activity of each sample was measured.

The effect of detergent on enzyme activity was examined using 0.05% (w/v) Span 80, Tween 80, and Triton X-100. Several solvents, including ethanol, methanol, acetone, isopropyl alcohol, 1-butanol, and toluene were added to the enzymatic reaction solution at a concentration of 2% (v/v) to find the optimum solvent for enzyme activity. The effect of ethanol concentration on enzyme activity was investigated by varying its concentration from 0 to 4% (v/v). To remove cofactors, urea was added to the purified enzyme at a final concentration of 3 M and then the enzyme was dialyzed at 4 °C for 24 h against 50 mM PIPES buffer (pH 6.5) [12]. This procedure has a negligible impact on enzyme activity. The reconstituted apoenzyme was concentrated to 10 mg/ml by ultrafiltration using the Centricon. After adding 0.2 mM FAD, FMN, NAD^+, or $NADP^+$ to the apoenzyme, the enzyme solution was incubated at 4 °C for 24 h. The effect of FAD concentration was investigated by varying the FAD concentration from 0 to 1.0 mM.

2.9. FAD assay

UV–visible absorption spectra of FAD, apoenzyme, and holoenzymes were measured from 300 to 600 nm using a Beckman Coulter DU 800 UV–visible spectrometer (Brea, CA, USA). Spectral measurements of the wild-type and variant enzymes were performed in 50 mM PIPES buffer (pH 6.5) containing 20 μM FAD and 1 mg/ml enzyme at 25 °C. After adding 1 mM FAD to 10 mg/ml wild-type and variant enzymes, the enzyme solutions were incubated at 4 °C for 16 h. The unbound FAD in the enzyme solutions was removed by untrafiltration using the Centricon (50 kDa cutoff), and the resulting solutions were heated at 100 °C for 15 min. After heating, the precipitated proteins were removed by centrifugation at 13,000 × *g* for 10 min, and the supernatant was used for the analysis of UV–visible spectra. The concentration of bound FAD to enzyme in the supernatant was determined using a linear calibration curve relating optical density at 450 nm and FAD concentration ranging from 0 to 150 μM (Supplementary Fig. 1).

2.10. Analytical methods

The enzymatic reaction solution was extracted with an equal volume of ethyl acetate. Solvent was removed from the extract using a rotary evaporator. The obtained fatty acids were silylated with a 2:1 mixture of pyridine and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide [13]. Silylated fatty acids in the organic phase were analyzed by a GC (Agilent 6890N, Santa Clara, CA, USA) equipped with a flame ionization detector and a Supelco SPB-1 capillary column. The column temperature was increased from 150 to 210 °C for 15 min and then maintained at 210 °C. The injector and detector were held at 260 and 250 °C, respectively. The hydroxy fatty acid products were identified by GC–MS (Agilent 5973N) with an electron impact ionization source. The ion source was operated at 70 eV and held at 230 °C.

3. Results

3.1. Gene cloning and molecular mass of the putative fatty acid hydratase

The *MCRA* gene (1770 bp), which was previously proposed as a fatty acid hydratase gene from *M. caseolyticus*, with the same sequence reported in GenBank (accession number NC_011999.1), was cloned and expressed in *E. coli*. The expressed enzyme was purified from the crude extract obtained from harvested cells by His-Trap HP affinity chromatography. The putative fatty acid hydratase from *M. caseolyticus* was purified with a purification of 9-fold, a yield of 38%, and a specific activity of 3300 unit/mg. The purified enzyme contained a hexa-histidine tag at the N terminus. The non-tagged enzyme was obtained from the removal of histidine tag of the histidine-tagged enzyme by treating thrombin. The specific activity of the non-tagged enzyme was almost the same as that of the histidine-tagged enzyme (Supplementary Table 1).

The purified protein showed a single band in SDS-PAGE with a molecular mass of approximately 68 kDa (Fig. 1A), which is consistent with the calculated value of 68.1 kDa based on the 589-residue amino acids plus the hexa-histidine tag. Based on the masses of the reference proteins, the native protein existed as a dimer with a molecular mass of 136 kDa as determined by gel filtration chromatography (Fig. 1B). Fatty acid hydratases from *E. meningoseptica*, *S. pyogenes*, and *B. breve* consisted of 646, 598, and 625 amino acid residues, respectively, and their subunit molecular masses were 66–70 kDa. The total molecular mass of fatty acid hydratase from *S. pyogenes* is 134 kDa as a dimer [8].

3.2. Identification of hydroxy fatty acid products formed by the putative fatty acid hydratase

The conversion of unsaturated fatty acid substrates into hydroxy fatty acid products catalyzed by the putative fatty acid hydratase from *M. caseolyticus* were analyzed using GC. The products were purified by solvent fractional crystallization at low temperature [11] and the purity of the products was greater than 99% as

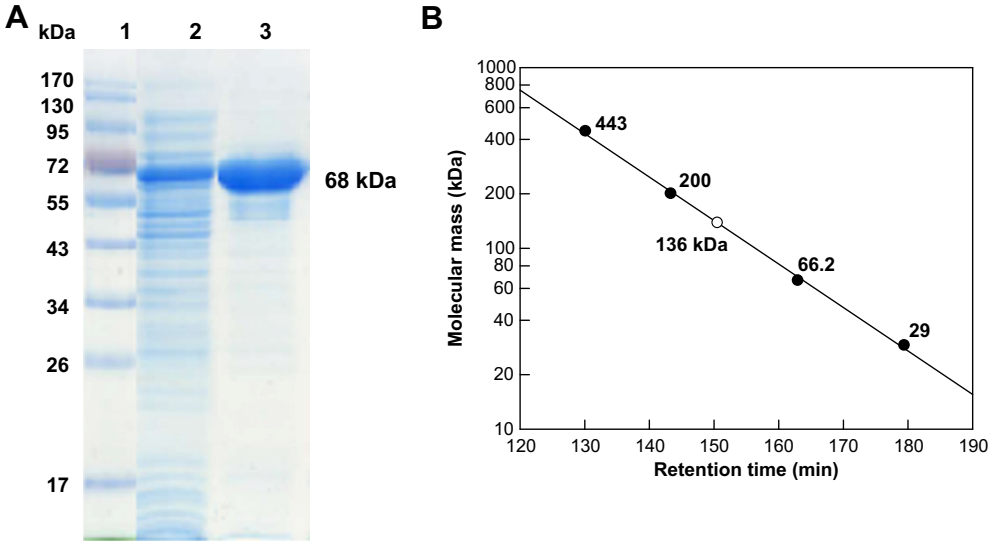

Fig. 1. SDS-PAGE analysis and molecular mass determination of the putative fatty acid hydratase from *M. caseolyticus*. (A) SDS-PAGE analysis. Lane 1, molecular mass marker proteins; lane 2, crude extract; lane 3, purified putative fatty acid hydratase. (B) Determination of molecular mass of the purified native enzyme by gel filtration chromatography. The reference proteins were apoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa).

determined by GC. The purified hydroxy fatty acids were used as the standard compounds after identification by GC–MS.

The silylated enzymatic product obtained from oleic acid gave the mass spectra shown in Fig. 2A. Peaks at *m*/*z* 229 and 331 were resulted from the cleavage of the hydroxyl group at the C10 position because of the loss of $C_{12}H_{27}OSi$ and C_8H_{17}, respectively, from the silylated hydroxy fatty acid, which is represented by peak at *m*/*z* 444. A peak at *m*/*z* 215 was formed by the loss of $C_{12}H_{25}O_2Si$. These

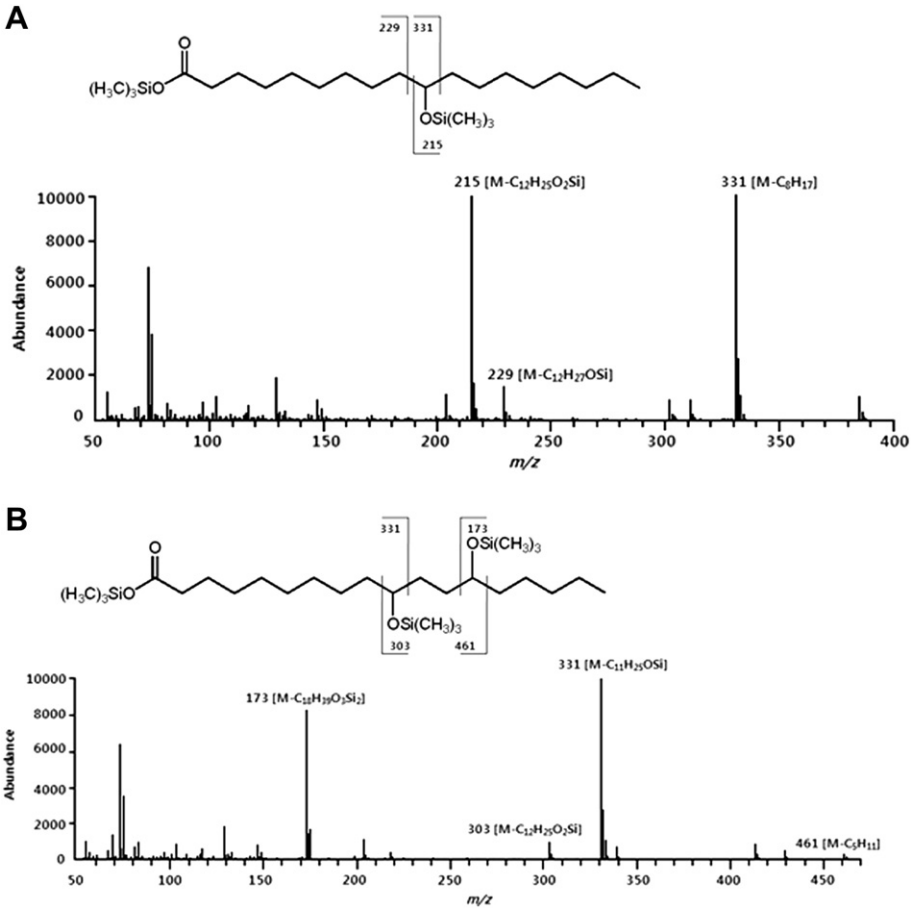

Fig. 2. Chemical structures and GC/MS spectra of the silylated (A) 10-hydroxyoctadecanoic acid and (B) 10,13-dihydroxyoctadecanoic acid products obtained from oleic acid and linoleic acid by the putative fatty acid hydratase from *M. caseolyticus*, respectively.

fragment peaks identified that the hydroxy fatty acid was a 10-hydroxyoctadecanoic acid. The mass spectra of the enzymatic fatty acid products formed from myristoleic acid, palmitoleic acid, linoleic acid, and α-linolenic acid also contained peaks at *m*/*z* 229 and 331, indicating that these compounds were 10-hydroxy fatty acids.

The mass spectrum of silylated dihydroxy fatty acid obtained from the enzymatic reaction for linoleic acid (Fig. 2B) contained peaks at *m*/*z* 303 and 331 and at 173 *m*/*z* and 461 resulting from the cleavage of the hydroxyl groups at the C10 and C13 positions, respectively. These results fragment peaks identified that the dihydroxy fatty acid was a 10,13-dihydroxyoctadecanoic acid. The dihydroxy fatty acid product formed from α-linolenic acid yielded a mass spectrum with peaks at *m*/*z* 301 and 331 and at *m*/*z* 171 and 461, indicating two hydroxyl groups at the C10 and C13 positions, respectively. The dihydroxy fatty acid was identified as a 10,13-dihydroxy-15(*Z*)-octadecenoic acid due to these fragment peaks. Peaks at *m*/*z* 227 and 329 in the mass spectrum of the hydroxy fatty acid formed from γ-linolenic acid also indicate a hydroxyl group at the C10 position. The mass spectrum of the silylated dihydroxy fatty acid obtained from γ-linolenic acid exhibits peaks at *m*/*z* 303 and 329 and at *m*/*z* 173 and 459 resulting from the cleavage of the hydroxyl group at C10 and C13 positions, respectively. The double bond between C6 and C7 of γ-linolenic acid caused a difference of 2 *m*/*z* units between the product peaks (Table 1). The mass spectrum indicated that the dihydroxy fatty acid was a 10,13-dihydroxy-6(*Z*)-octadecenoic acid.

3.3. Conversion of unsaturated fatty acids into hydroxy fatty acids by the putative fatty acid hydratase

The enzymatic conversion of oleic acid was examined under the standard conditions described above. After 840 min, the conversion of oleic acid to 10-hydroxyoctadecanoic acid occurred with a molar yield of 98% (Fig. 3A). The enzymatic reaction did not occur with 2 mM 10-hydroxyoctadecanoic acid as a substrate. Thus, the enzymatic reaction was irreversible (Fig. 3B). The enzyme also converted myristoleic acid, palmitoleic acid, and oleic acid into 10-hydroxytetradecanoic acid, 10-hydroxyhexadecanoic acid, and 10-hydroxyoctadecanoic acid, respectively, with no other byproducts (Supplementary Fig. 2A, B, and C). Linoleic acid, α-linolenic acid, and γ-linolenic acid were each converted into the two products 10-hydroxy-12(*Z*)-octadecenoic acid and 10,13-dihydroxyoctadecanoic acid, 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid and 10,13-dihydroxy-15(*Z*)-octadecenoic acid, and 10-hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid and 10,13-dihydroxy-6(*Z*)-octadecenoic acid, respectively (Supplementary Fig. 2D, E, and F).

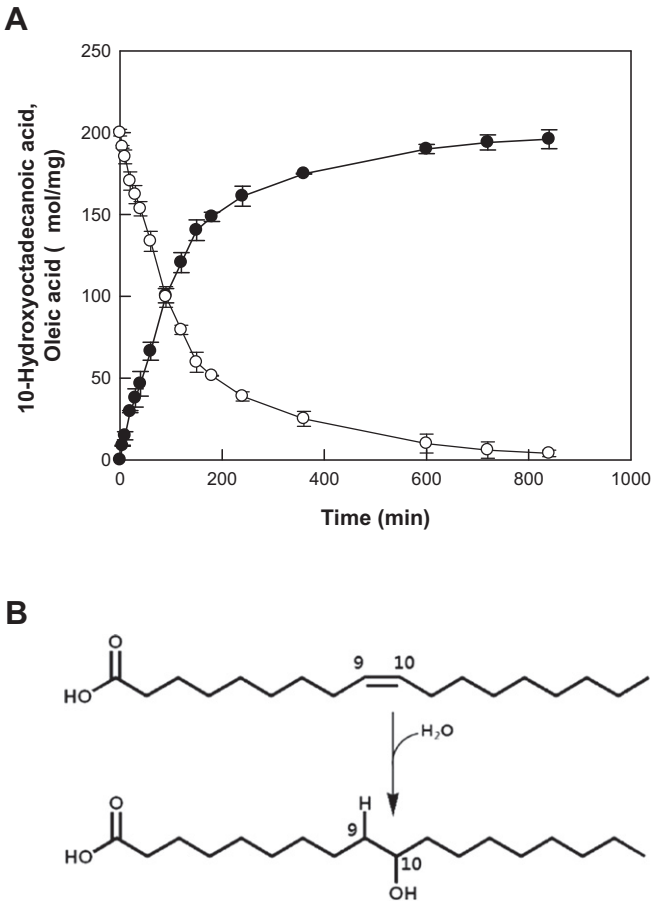

Fig. 3. Irreversible catalytic reaction of oleic acid into 10-hydroxyoctadecanoic acid by oleate hydratase from *M. caseolyticus*. (A) Time-course reaction. The conversion reactions of oleic acid (○) into 10-hydroxyoctadecanoic acid (●). The reactions were performed by varying the reaction time in 50 mM PIPES buffer (pH 6.5) containing 2 mM oleic acid and 33 unit/ml enzyme at 25 °C for 840 min. Data represent the means of three experiments and error bars represent standard deviation. (B) Reaction mechanism. The oleate hydratase hydrated oleic acid at its *cis*-9-double bond to yield 10-hydroxyoctadecanoic acid.

3.4. Identification of the putative fatty acid hydratase

The control experiment for the enzymatic reaction was performed with heat-inactivated enzyme or without the enzyme under the same reaction conditions. In the experiment, no hydroxy fatty acids were detected. The substrate specificity of the putative fatty hydratase was evaluated by determining the concentrations of

Table 1
Specific activity and kinetic parameters of the putative fatty acid hydratase from *M. caseolyticus* for fatty acids as substrates.

Substrate	Product(s)	GC retention time (min)	GC/MS fragments (*m*/*z*)	Specific activity (unit/mg)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mm^{-1} min^{-1})
Myristoleic acid	10-Hydroxytetradecanoic acid	7.15	388(M), 331, 229, 159	18 ± 0.2	300 ± 7	2 ± 0.0	8 ± 0.2
Palmitoleic acid	10-Hydroxyhexadecanoic acid	10.26	416(M), 331, 229, 187	1680 ± 8	570 ± 9	230 ± 4	390 ± 6
Oleic acid	10-Hydroxyoctadecanoic acid	13.76	444(M), 331, 229, 215	3300 ± 12	340 ± 2	470 ± 1	1360 ± 5
Linoleic acid	10-Hydroxy-12(*Z*)-octadecenoic acid	13.39	442(M), 331, 229, 213	58 ± 0.9	390 ± 1	8 ± 0.2	20 ± 0.4
	10,13-Dihydroxyoctadecanoic acid	13.60	532(M), 461, 331, 303, 173	5 ± 0.8	340 ± 2	1 ± 0.1	3 ± 0.6
α-Linolenic acid	10-Hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid	13.45	440(M), 331, 229, 211	59 ± 2.9	320 ± 2	8 ± 0.2	25 ± 0.6
	10,13-Dihydroxy-15(*Z*)-octadecenoic acid	13.65	530(M), 461, 331, 301, 171	17 ± 0.9	330 ± 4	2 ± 0.6	8 ± 0.2
γ-Linolenic acid	10-Hydroxy-6(*Z*),12(*Z*)-octadecadienoic acid	12.72	440(M), 329, 227, 213	1120 ± 1.2	590 ± 2	160 ± 1	270 ± 0.1
	10,13-Dihydroxy-6(*Z*)-octadecenoic acid	12.84	530(M), 459, 329, 303, 173	90 ± 0.9	600 ± 4	12 ± 0.2	21 ± 0.5

ND, kinetic parameters are not detected by the analytical methods used in this study.
Data represent the mean of three experiments and ± values represent standard deviations.

the reaction products by GC and then identifying the products by GC–MS. No enzymatic activity was observed for lauric acid (C12), myristic acid (C14), palmitic acid (C16), stearic acid (C18), arachidic acid (C20), petroselinic acid (C18:1$^{\Delta 6Z}$), elaidic acid (C18:1$^{\Delta 9E}$), vaccenic acid (C18:1$^{\Delta 11Z}$), conjugated linoleic acids (C18:2$^{\Delta 9E,11E}$, C18:2$^{\Delta 9Z,11E}$, and C18:2$^{\Delta 10E,12Z}$), arachidonic acid (C20:4$^{\Delta 5Z,8Z,11Z,14Z}$), erucic acid (C22:1$^{\Delta 13Z}$), or nervonic acid (C22:1$^{\Delta 15Z}$). However, the enzyme exhibited some activity for myristoleic acid (C14:1$^{\Delta 9Z}$), palmitoleic acid (C16:1$^{\Delta 9Z}$), oleic acid (C18:1$^{\Delta 9Z}$), linoleic acid (C18:2$^{\Delta 9Z,12Z}$), α-linolenic acid (C18:3$^{\Delta 9Z,12Z,15Z}$), and γ-linolenic acid (C18:3$^{\Delta 6Z,9Z,12Z}$). These results indicate that the putative fatty acid hydratase had no specificity for saturated fatty acids and *trans*-, *cis*-5-, *cis*-6-, *cis*-8-, *cis*-11-, *cis*-13-, *cis*-14-, and *cis*-15-double bond unsaturated fatty acids, but had for *cis*-9- and/or *cis*-12-double bond unsaturated fatty acids without *trans*-configurations. Thus, *cis*-9 unsaturated fatty acids were converted into 10-hydroxy fatty acids as a single product, *cis*-9 and *cis*-12 unsaturated fatty acids were converted into the two products 10-hydroxy fatty acids and 10,13-dihydroxy fatty acids.

The specific activity, k_{cat}, and k_{cat}/K_m of the enzyme followed the order oleic acid > palmitoleic acid > γ-linolenic acid (the faster product) > γ-linolenic acid (the slower product) > α-linolenic acid (the faster product) > linoleic acid (the faster product) > myristoleic acid > α-linolenic acid (the slower product) > linoleic acid (the slower product) (Table 1). The faster and slower products were 10-hydroxy fatty acids and 10,13-dihydroxy fatty acids, respectively. The enzyme exhibited the highest catalytic efficiency (k_{cat}/K_m) for oleic acid among the substrates tested. The k_{cat}/K_m for oleic acid (1359 mM^{-1} min^{-1}) was 3.5-fold higher than that for the second favored substrate palmitoleic acid (387 mM^{-1} min^{-1}). These results indicate that the putative fatty acid hydratase was an oleate hydratase.

3.5. Effects of reaction conditions on the activity of the oleate hydratase

The purified enzyme obtained after the removal of metal ions by treating 10 mM EDTA exhibited 90% activity of the native enzyme activity. The addition of EDTA to oleate hydratase from *E. meningoseptica* had no effect on enzyme activity [10]. After adding divalent metal ions to the EDTA-treated oleate hydratase, the activity was reduced to 25–44% of the native enzyme activity, following the order $Mg^{2+} > Ca^{2+} > Co^{2+} > Mn^{2+} > Ni^{2+} > Ba^{2+} > Zn^{2+} > Fe^{2+}$ (Supplementary Fig. 3A). The addition of Cu^{2+} resulted in no activity. Maximum enzyme activity was observed at pH 6.5 and 25 °C (Fig. 4A and B). The effect of temperature on enzyme stability was investigated. After 120 min, the enzyme activity nearly unchanged at 25 °C, but was reduced to only 25% at 35 °C (Supplementary Fig. 3B). Enzyme activity was decreased with the addition of any detergent (Supplementary Fig. 3C). Among the solvents tested, ethanol was the most beneficial for enzyme activity (Supplementary Fig. 3D), with an optimal concentration of 2% (v/v) (Fig. 4C). The addition of the cofactor FMN, NAD^+, or $NADP^+$ did not affect enzyme

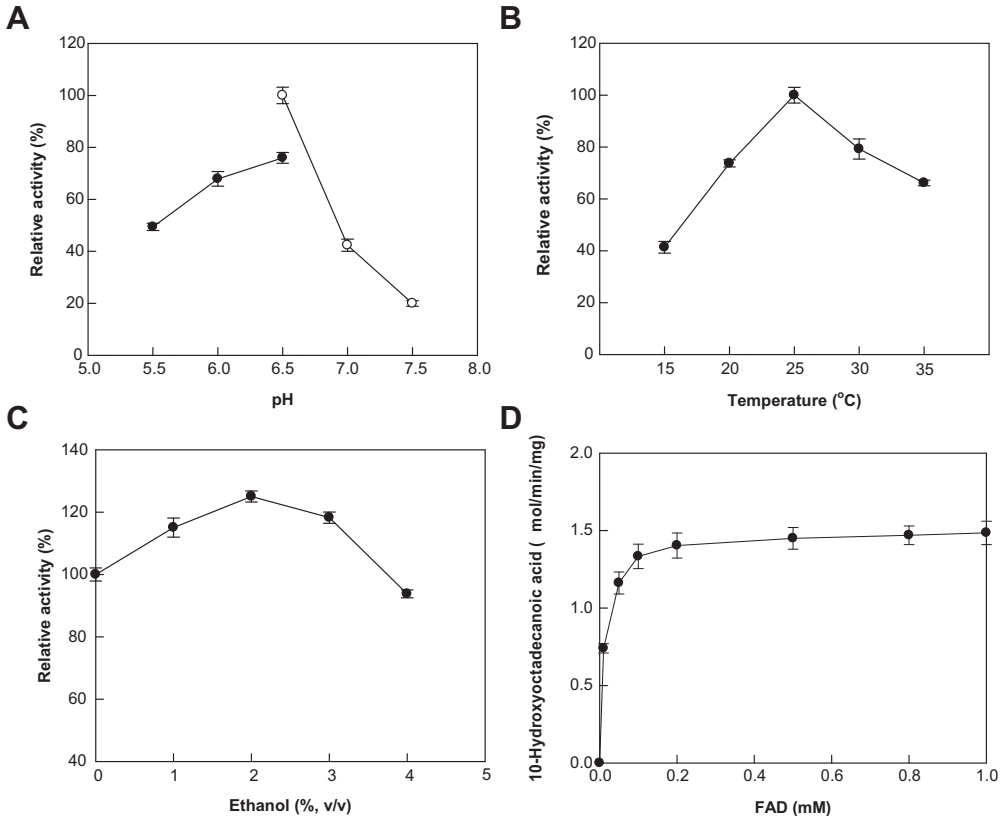

Fig. 4. Effects of pH, temperature, and concentrations of ethanol and FAD on the activity of oleate hydratase from *M. caseolyticus*. (A) Effect of pH. The reactions were performed in 50 mM MES buffer for pH 5.5 to 6.5 (●) and 50 mM PIPES buffer for pH 6.5 to 7.5 (○) with 2 mM oleic acid and 33 unit/ml enzyme at 25 °C for 10 min. (B) Effect of temperature. The reactions were performed in 50 mM PIPES buffer (pH 6.5) containing 2 mM oleic acid and 33 unit/ml enzyme for 10 min in temperature range of from 15 to 35 °C. (C) Effect of ethanol concentration. The reactions were performed by varying the concentration of ethanol in 50 mM PIPES buffer (pH 6.5) containing 2 mM oleic acid and 33 unit/ml enzyme at 25 °C for 10 min. (D) Effect of FAD concentration. The reactions were performed by varying the concentration of FAD in 50 mM PIPES buffer (pH 6.5) containing 2 mM oleic acid and 33 unit/ml enzyme at 25 °C for 10 min. 0 mM FAD indicates the apoenzyme without the addition of FAD. Data represent the means of three experiments and error bars represent standard deviation.

activity. But, when FAD was not added to the apoenzyme, all catalytic activity was abolished (Fig. 4D), and enzyme activity increased with increasing FAD concentration, reaching a plateau above 0.2 mM.

The maximum activity of oleate hydratase from *M. caseolyticus* was observed at pH 6.5 and 25 °C with 2% (v/v) ethanol and 0.2 mM FAD. The maximum activity of *E. meningoseptica* oleate hydratase occurred at pH 6.0 with 5% isopropyl alcohol and 50 mM NaCl [10]. However, the effects of temperature and solvent were not reported. The reaction conditions of *S. pyogenes* fatty acid hydratase were reported to be pH 6.1, 37 °C, 2% ethanol, and 0.02 mM FAD [8]. The reaction of *B. breve* fatty acid hydratase was performed at pH 7.5 and 25 °C [9].

3.6. Spectral and kinetic analyses of FAD-binding residues in the oleate hydratase

The wild-type holoenzyme exhibited two peaks at 375 and 445 nm as a typical UV–visible absorption spectrum of an FAD-dependent enzyme. These peaks were not present in the absorption spectrum of the apoenzyme obtained after the removal of FAD (Supplementary Fig. 4). The five conserved residues of G29, G31, S34, E50, and E56 in the FAD-binding motif of fatty acid hydratases (Supplementary Fig. 5) were individually replaced with alanine. The UV–visible absorption spectra of S34A and E50A displayed reduced peaks at 375 and 445 nm relative to those of the wild-type enzyme (Fig. 5A, D, and E), and the FAD-dependent peaks of G29A, G31A, and E56A disappeared (Fig. 5B, C, and F). The heat precipitation was performed to release FAD in the enzyme. The UV–visible absorption spectra of the supernatant of the wild-type and variant enzymes after heat precipitation were similar to those of the enzymes before heat precipitation (Supplementary Fig. 6). These results mean that a non-covalently bound FAD is fully released in the solution upon protein denaturation by heat. After heat precipitation, the FAD concentration of the supernatant of the wild-type was 141 μM in 145 μM wild-type enzyme (Fig. 6), indicating that the coenzyme FAD was bound to apoenzyme with a ratio of 1:1. The bound FAD concentrations followed the order wild-type enzyme (141 μM) > E50A (98 μM) > S34A (75 μM) > E56A (25 μM) > G29A (19 μM) > G31A (8 μM).

The specific activities and k_{cat}/K_m values of the S34A and E50A variants for oleic acid were 60–85% those of the wild-type enzyme (Table 2). The affinity and k_{cat} of these variants for oleic acid were also lower than those of the wild-type enzyme. Alanine-substitution of G29, G31, and E56 resulted in loss of enzyme activity.

4. Discussion

The MCRA protein family is widely distributed in bacteria, especially pathogenic and intestinal bacteria. MCRA was found first in a strain of *S. pyogenes* that causes human pathogenesis, including acute rheumatic fever and heart tissue damage [14]. The MCRA of pathogenic bacteria can survive in blood with the aid of human keratinocytes for adherence and internalization [8] and are less sensitive to heat and solvent stresses [9,15]. The MCRA of intestinal bacteria is involved in anti-microbial [3,16] and anti-stress effects [17]. Many bacteria are sensitive to fatty acids, and unsaturated fatty acids are much more toxic than saturated fatty acids [18,19].

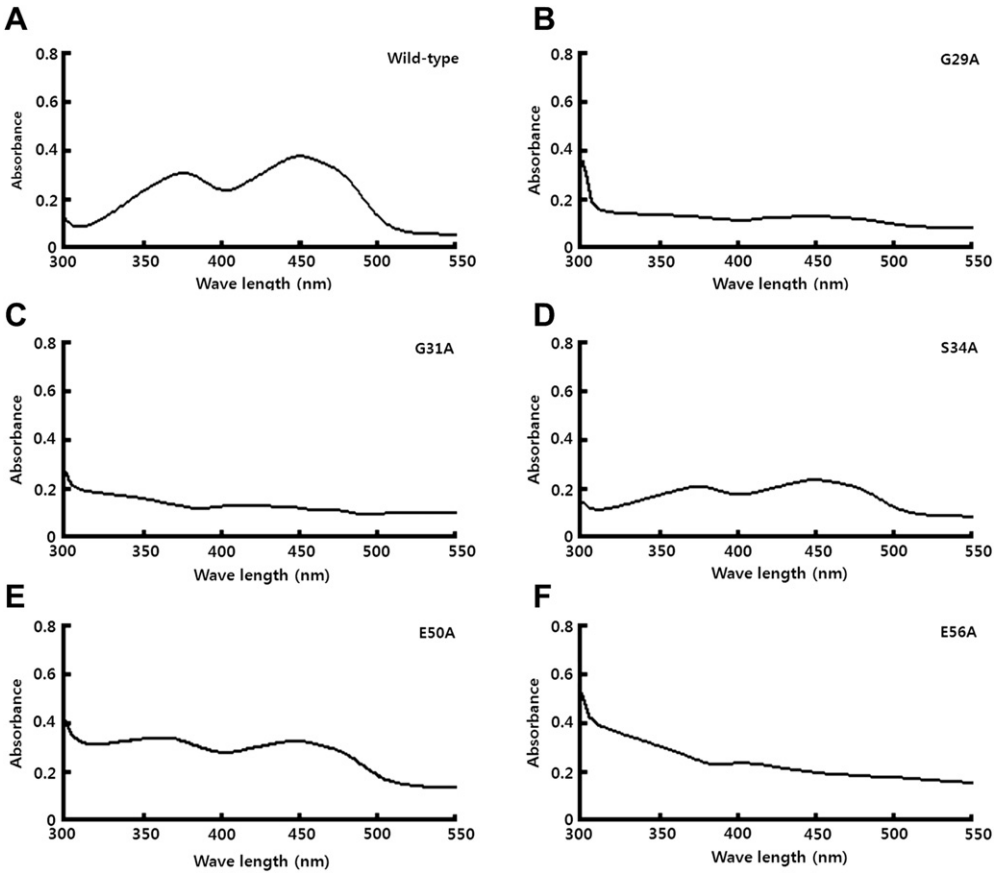

Fig. 5. UV–visible absorption spectra of FAD-containing wild-type, and variant enzymes. (A) Wild-type. (B) G29A. (C) G31A. (D) S34A. (E) E50A. (F) E56A. The concentrations of the wild-type and variant enzymes were approximately 1 mg/ml in 50 mM PIPES buffer (pH 6.5).

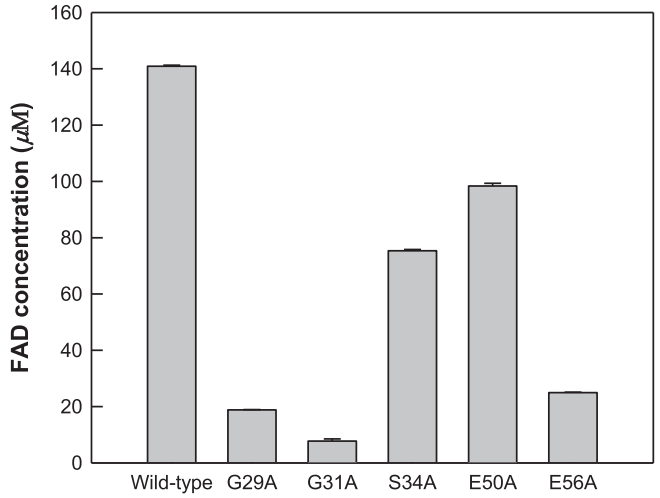

Fig. 6. Concentrations of bound FAD to the wild-type and variant enzymes. The concentrations of the wild-type and variant enzymes were approximately 10 mg/ml in 50 mM PIPES buffer (pH 6.5). After heat precipitation, the FAD concentration of the supernatant was measured at 450 nm of optical density.

Unsaturated fatty acids inhibit the development of cellular membranes because their kinked structure disrupts the lipid bilayer formation [20] and inhibits enoyl-ACP reductase, which involved in bacterial fatty acid synthesis [21]. The hydration of unsaturated fatty acids is suggested to be a detoxification mechanism in bacteria harboring MCRA proteins and a survival strategy for living in fatty acid-rich environments [8]. Thus, MCRA proteins are fatty acid hydratases.

The amino acid sequence of oleate hydratase from *M. caseolyticus* exhibited 57, 55, and 41% identity with the characterized fatty acid hydratases from *S. pyogenes* [8], *B. breve* [9], and *E. meningoseptica* [10], respectively, and showed 11, 10, and 10% identity with the other oleate-converting enzymes P450 monooxygenase from *B. licheniformis* [22], oleate hydroxylase from the plant *R. communis L* [6], and lipoxygenase from *Pseudomonas* 42A2 [7], respectively. These results indicate that the oleate hydratase is much more closely related to fatty acid hydratases than to other oleate-converting enzymes.

The substrate specificity of fatty acid hydratases for fatty acids was not investigated quantitatively and the name of the specific hydratase was not defined because the hydroxy fatty acid products of the previously reported fatty acid hydratases are not commercially available and have not been isolated [8–10]. In the present study, all of the hydroxy fatty acids produced by the putative fatty acid hydratase from *M. caseolyticus* were purified, and the purified products (>99%) (Supplementary Fig. 2) were identified by GC–MS (Fig. 2, Table 1) and used as standards in subsequence analyses. Based on the quantitative determination of the substrate specificity, the putative fatty acid hydratase was identified as an oleate hydratase. The enzyme exhibited hydration activity for myristoleic acid, palmitoleic acid, oleic acid, linoleic acid, and α- and γ-linolenic acids. Fatty acid hydratase from *S. pyogenes* recognized specifically palmitoleic acid, oleic acid, linoleic acid, α-linolenic acid but not myristoleic acid and γ-linolenic acid [8]. Fatty acid hydratase from *B. breve* showed activity for palmitoleic acid, oleic acid, linoleic acid, but not for myristoleic acid and α-linolenic acid [9]. The hydration activity of this enzyme for γ-linolenic acid was not investigated. The enzyme from *Flavobacterium* sp. DS5 involved in the hydration of unsaturated fatty acids was suggested to be a hydratase [23,24]. *Flavobacterium* sp. DS5 [24] and *Stenotrophomonas nitritireducens* [25] had hydration activities for myristoleic acid, palmitoleic acid, oleic acid, linoleic acid, and α- and γ-linolenic acids, indicating that the substrate specificity of these bacteria is the same as that of oleate hydratase from *M. caseolyticus*. Unfortunately, the different specificity of the fatty acid hydratases and bacteria cannot be elucidated because the crystal structure and active site of hydratase have not been determined.

It has been suggested that the conversion of oleic acid into 10-hydroxyoctadecanoic acid using crude extract from *Pseudomonas* sp. strain 3266 is a reversible reaction [10,26]. This putative enzyme involved in the study was referred to as an oleate hydratase, although the enzyme was not purified and characterized. The strain converted oleic acid into not only 10-hydroxyoctadecanoic acid but also 10-oxo-octadecanoic acid, suggesting that other fatty acid-converting enzymes must be present in the crude extract. In the present study, the purified oleate hydratase did not convert 10-hydroxyoctadecanoic acid into oleic acid. The conversion of oleic acid with D_2O and/or $H_2^{18}O$ by *Nocardia cholesterolicum* [27] and *Pseudomonas* sp [28]. showed the incorporation of D at the C9 position and ^{18}O at the C10 position of 10-hydroxyoctadecanoic acid. Therefore, oleate hydratase catalyzes the irreversible addition of a hydrogen atom and a hydroxy group from water to the carbon–carbon double bond at the C9 and C10 positions, respectively, to make 10-hydroxyoctadecanoic acid (Fig. 3B).

Spectral analyses [29] and kinetic analyses of variants [12] have been widely used for the characterization of dinucleotide-dependent enzymes. When FAD was not bound to the enzyme, its catalytic activity was abolished (Fig. 4D). These results indicate that the oleate hydratase from *M. caseolyticus* is FAD-dependent. All members of the glutathione reductase (GR) family, including fatty acid hydratases, contain a FAD-binding motif such as a $GXGXXS_{(A/G)}X_{15}E_{(D)}X_5E$ (where X denotes any amino acid) sequence at the N terminus [30,31]. The roles in FAD-binding for the five conserved residues of G29, G31, S34, E50, and E56 in the FAD-binding motif were investigated. The absorption peaks at 375 and 445 nm, bound FAD amounts, specific activities, and k_{cat} values of S34A and E50A were all reduced by alanine-substitution (Figs. 5 and 6, and Table 2). G29A, G31A, and E56A exhibited no adsorption peaks and no enzyme activity. The bound FAD concentrations of S34A and E50A were more than 50% of the wild-type enzyme FAD, whereas those of G29A, G31A, and E56A were less than 20%. Mutations at G29, G31, and E56 resulted in significantly reduced molecular interactions with FAD and resulted in no activity. Thus, G29, G31, and E56 are essential for FAD-binding and S34 and E50 are involved in FAD-binding. UV–visible absorption spectra of the holoenzyme of *M. caseolyticus* oleate hydratase were measured at 30 s intervals for 10 min (Supplementary Fig. 7A). The spectra changed very little, indicating that the oxidation–reduction reaction of FAD did not occur. To investigate the effect of the addition of FAD on enzyme stability, the reactions were performed after incubating at 25 °C for 24 h with 1 mg/ml purified enzyme in the presence and absence of

Table 2
Kinetic parameters of the wild-type and variant enzymes in the FAD-binding motif of oleate hydratase from *M. caseolyticus*.

Enzyme	Specific activity (unit/mg)	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild-type	3300 ± 54	340 ± 2	470 ± 1	1360 ± 5
G29A	ND	ND	ND	ND
G31A	ND	ND	ND	ND
S34A	2540 ± 25	380 ± 5	300 ± 4	800 ± 1
E50A	2820 ± 36	390 ± 7	440 ± 5	1140 ± 2
E56A	ND	ND	ND	ND

ND, kinetic parameters are not detected by the analytical methods used in this study.
Data represent the mean of three experiments and ± values represent standard deviations.

1 mM FAD (Supplementary Fig. 7B). The production of 10-hydroxystearic acid from oleic acid by the enzyme in the absence of FAD was 7-fold lower than that in the presence of 1 mM FAD. Thus, FAD in the oleate hydratase does not seem to be involved in the hydration reaction itself but rather in the structural stabilization of the protein.

In the present study, the putative fatty acid hydratase from *M. caseolyticus* was identified as an oleate hydratase by characterizing the quantitative biochemical properties of the enzyme. The enzyme exhibited hydration activity only for *cis*-9- and/or *cis*-12-double bond unsaturated fatty acids without *trans*-configurations, irreversibly producing 10-hydroxy fatty acids and 10,13-dihydroxy fatty acids. The oleate hydratase was FAD-dependent, and G29, G31, and E56 were essential for FAD-binding.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biochi.2011.12.011.

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