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Production of hydroxy fatty acids by microbial fatty acid-hydroxylation enzymes

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ABSTRACT

Hydroxy fatty acids are widely used in chemical, food, and cosmetic industries as starting materials for the synthesis of polymers and as additives for the manufacture of lubricants, emulsifiers, and stabilizers. They have antibiotic, anti-inflammatory, and anticancer activities and therefore can be applied for medicinal uses. Microbial fatty acid-hydroxylation enzymes, including P450, lipoxygenase, hydratase, 12-hydroxylase, and diol synthase, synthesize regio-specific hydroxy fatty acids. In this article, microbial fatty acid-hydroxylation enzymes, with a focus on region-specificity and diversity, are summarized and the production of mono-, di-, and tri-hydroxy fatty acids is introduced. Finally, the production methods of regio-specific and diverse hydroxy fatty acids, such as gene screening, protein engineering, metabolic engineering, and combinatory biosynthesis, are suggested.

Contents

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1. Introduction

Hydroxy fatty acids are saturated or unsaturated fatty acids, consisting of one or more hydroxyl groups and a long chain unbranched carbon chain with a carboxyl group at one end. They occur in nature as components of cerebrosides (Kishimoto and Radin, 1963), triacylglycerols (Bafor et al., 1991), waxes (Jetter and Kunst, 2008), and other lipids (Kock et al., 2003) in animals, plants, and microorganisms. Hydroxy fatty acids have become the focus of a great deal of attention because they are important materials in chemical, food, and cosmetic industries (Hou, 2000) as well as crucial signaling compounds related to human disease (Joo and Oh, 2012).

Fatty acid-hydroxylation enzymes from animals, plants, and microorganisms produce hundreds of hydroxy fatty acids. 12-Hydroxylase (van de Loo et al., 1995), lipoxygenase (Oliw, 2002), and cytochrome P450 monooxygenase (P450) (Kandel et al., 2006) from plants produce ricinoleic acid (12-OH 18:1$^{\Delta 9Z}$) and 12-hydroxystearic acid (12-OH 18:0), 13-hydroxyoctadecadienoic acid (HODE), and ω-hydroxy fatty acid, respectively. Hydratase (Joo et al., 2012a), 12-hydroxylase (Morris et al., 1966), P450 (Kitazume et al., 2008), and diol synthase (Brodowsky et al., 1992) from microorganisms produce 10-hydroxystearic acid, ricinoleic acid, ω-hydroxy fatty acid, and di-hydroxy fatty acid, respectively. Microbial and plant hydroxy fatty acids have been used as starting materials for the synthesis of polymers and as additives for the manufacture of lubricants, emulsifiers, and stabilizers (Mutlu and Meier, 2010; Ogunniyi, 2006). Lipoxygenase from animals converts arachidonic acid (20:4$^{\Delta 5Z,8Z,11Z,14Z}$) to leukotriene and lipoxin, which are signaling compounds involved in the regulation of inflammation (Funk, 2001). Microbial hydroxy fatty acids are more regio-specific and diverse than plant and animal hydroxy fatty acids because hydratase and diol synthase are only found in microorganisms. Microbial hydroxy fatty acids are also signaling compounds involved in host–pathogen interactions in fungi (Tsitsigiannis and Keller, 2007) and bacteria (Vance et al., 2004). They exhibit potent pharmaceutical activities, including anti-bacterial, anti-fungal, and anti-diabetic activities (Bajpai et al., 2009; Hou, 2009; Paul et al., 2010). Thus, hydroxy fatty acids can be used to the wide range of industrial and medicinal applications. However, hydroxy fatty acids produced by microbial enzymes have been studied less than those produced by plant and animal enzymes.

Individual fatty acid-hydroxylation enzymes have been reviewed (Andreou et al., 2009; Brash, 1999; Buczynski et al., 2009; Ivanov et al., 2010; Joo and Oh, 2012; Mosblech et al., 2009; Oliw, 2002; Van Bogaert et al., 2011). However, integrated reviews on fatty acid-hydroxylation enzymes have not been published. In this review, fatty acid-hydroxylation enzymes for the production of regio-specific and diverse hydroxy fatty acids are described.

2. Uses and applications of hydroxy fatty acids

2.1. Industrial uses and applications

Hydroxy fatty acids have a higher reactivity, solvent miscibility, stability, and viscosity, compared to non-hydroxylated fatty acids (Metzger and Bornscheuer, 2006). Thus, they are widely used in chemical, food, and cosmetic industries. The industrial and medicinal uses and properties of hydroxy fatty acids, including mono-, di-, and trihydroxy fatty acids, are summarized in Table 1. 12-Hydroxystearic acid is mainly used as a thickening agent for the manufacture of lubricating greases. 10-Hydroxystearic acid, with properties similar to those of 12-hydroxystearic acid, is used in manufacturing lubricants (Hou, 2000, 2009). Ricinoleic acid is used in making soaps and in textile finishing (Mutlu and Meier, 2010; Ogunniyi, 2006).

Hydroxy fatty acids are used as starting materials for the synthesis of resins, nylons, polyurethanes, plastics, lubricants, biopolymers, and soaps (Mutlu and Meier, 2010; Ogunniyi, 2006). The polymers synthesized from hydroxy fatty acids have some advantages over petroleum-derived polymers, such as higher resistance to heat, chemicals, and impact, more flexibility, higher biocompatibility, and no toxicity (Burdock et al., 2006). They are used in a variety of consumer products, including clothes, shoes, cosmetic ingredients, shampoos, food and drug containers, medicinal tubing and devices, drug delivery, and interior materials. Hydroxy fatty acids have been used as additives for the manufacture of plasticizers, emulsifiers, stabilizers, grease, paint, and coating. They have been applied to renewable energy sources for biodegradable oil-based lubricants and biodiesel (Lima Da Silva et al., 2006). Hydroxy fatty acids are also used as precursors in the production of flavor and fragrance lactones by bacteria and yeast (Endrizzi et al., 1996; Waché et al., 2003, 2006). Therefore, the industrial uses of hydroxy fatty acids, as environment-friendly and sustainable technology for the future, offer potential ways to solve current energy resource crises by replacing petroleum oil.

2.2. Medicinal uses and applications

Plant 9- and 13-lipoxygenases convert linoleic acid (18:2$^{\Delta 9Z,12Z}$) to 9- and 13-HODE, respectively, and animal 15-lipoxygenase converts arachidonic acid to 15-hydroxyeicosatetraenoic acid (HETE). These compounds have antifungal, anti-inflammatory, and anticancer activities (Itoh et al., 2008; Moreno, 2009; Shureiqi et al., 2003). 10-HODE obtained from oleic acid (18:1$^{\Delta 9Z}$) by microorganisms shows inhibitory activity toward α-glucosidase (Paul et al., 2010). Ricinoleic acid has a laxative effect and anti-inflammatory activity (Tunaru et al., 2012). 14,21-Dihydroxy-docosahexanoic acid (DHA) is used to treat the wounds of diabetics (Tian et al., 2011), and 9,12,13-trihydroxy-octadecenoic acid (TODE) is used as an adjuvant for vaccines (Nagai et al., 2010). Lipoxins derived from arachidonic acid and resolvins derived from eicosapentaenoic acid (EPA, 20:5 $^{\Delta 5Z,8Z,11Z,14Z,17Z}$) are anti-inflammatory and pro-resolving lipid mediators (Schwab and Serhan, 2006; Serhan et al., 2008). These compounds protect cells from degeneration and reduce the use of antibiotics (Chiang et al., 2012; Stables and Gilroy, 2011). The spread for antibiotics resistance is an important issue. Lipid mediators have been suggested as an alternative treatment against bacterial infection to reduce the use of antibiotics.

The specific inhibitors of fatty acid-hydroxylation enzymes, such as lipoxygenase and P450, can be used as selective drugs for treating cancer, arthritis, cardiovascular disease, metabolic syndromes, and Alzheimer's disease. Thus, their discovery contributes to human health, especially to relief from pain and inflammation (Schneider and Pozzi, 2011). Market sales related to pain and inflammation in 2001 accounted for US $25 billion (Renfrey et al., 2003), and they are steadily increasing.

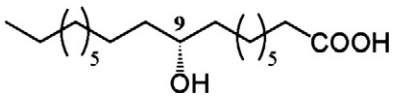
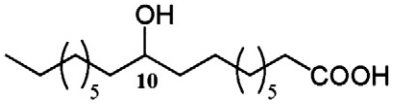
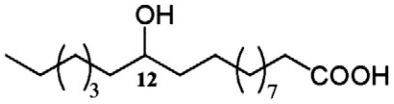
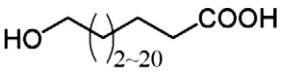
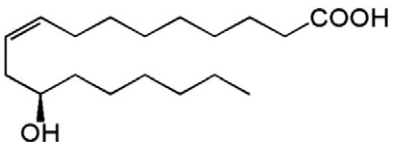
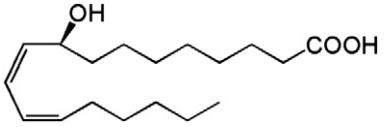
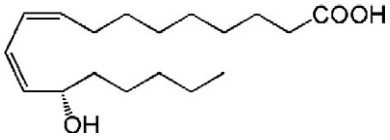
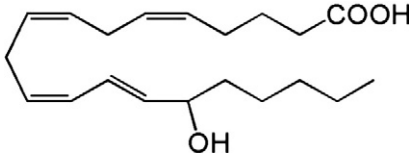
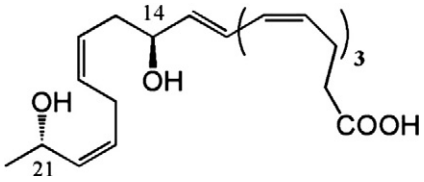
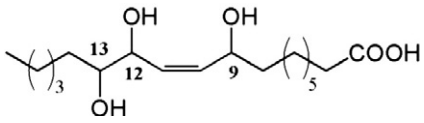
3. Fatty acid mono-hydroxylation enzymes

3.1. P450 monooxygenases

With NAD(P)H as a cofactor, P450s catalyze the insertion of one oxygen atom from molecular oxygen into an organic substrate through electron transfer (Fig. 1a). Fatty acid-hydroxylation P450s are classified into carboxyl-terminal hydroxylases (α-hydroxylases) and terminal or subterminal hydroxylases (ω-hydroxylases) (Van Bogaert et al., 2011) (Table 2). The ω-hydroxylases (EC 1.14.15.3) are CYP2 and 4 in animals (Capdevila et al., 1996; Fer et al., 2008); CYP76, 78, 86, 92, 94, 96, and 97 in plants (Pinot and Beisson, 2011); and CYP102, 105, 107, 109, and 153 in bacteria (Fabritius et al., 1996). The only α-hydroxylase (EC 1.11.2.4) is CYP152 in plant and bacteria (Hamberg et al., 1999; Matsunaga et al., 1999).

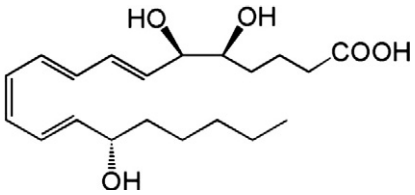
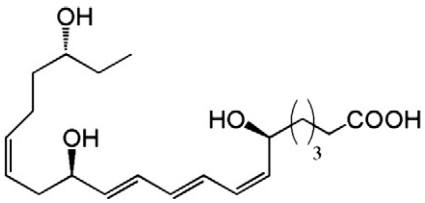
Among the ω-hydroxylases, the hydroxylation activity of yeast CYP52 toward C_{12}–C_{18} fatty acids, which are converted to ω-hydroxy

Table 1
Uses and properties of hydroxy fatty acids.

HFA	Usage or status	Feature	Reference
9*R*-Hydroxystearic acid 	– Stereo-selective inhibitor against human histone deacetylase I (colon cancer) – Apoptosis inducer and regulation of cell division	– Endogenous by-product of lipid peroxidation	Parolin et al. (2012)
10-Hydroxystearic acid 	– Lubricant – Precursor of flavor (lactone)	– Similar physico-chemical properties with 12-HSA	Joo and Oh (2012)
12-Hydroxystearic acid 	– Acrylic polymer, rubber, wax, and grease – Coating (food container, drug, car, and variant) – Hydrophobic drug delivery – Cosmetics and personal care (Emollient) – Shrink resistant for wool, leather softener	– High melting point, thermostable, and impact strength. – Gel state at room temperature – Safety (no toxic or hazardous)	Gow (2010)
ω-Hydroxy fatty acids 	– Polymeric monomer of plastic manufacture – Antibacterial, antifungal, and anticancer	– Polyester-like property, degradable	Metzger and Bornscheuer (2006)
Recinoleic acid 	– Polyester polyols, polyurethane, and plasticizer – Emulsifier, lubricant, and dispersant – Precursor of flavor and fragrance – Laxatives, drug delivery, food additives, and personal care	– Advanced flexibility, heat, chemical resistance than petroleum derived polymers. – Biocompatibility and degradability – Safety (no toxic or hazardous)	Biermann et al. (2011), Tunaru et al. (2012)
9*S*-HODE 	– Increased in atherosclerosis as major component of oxidized low density lipoprotein and also increased in rheumatoid arthritis – Antifungal activity	– PPAR-γ ligand (agonist) – G2A receptor ligand under stress	Itoh et al. (2008), Jira et al. (1997)
13*S*-HODE 	– Inhibition of cancer cell adhesion – Reduce artherogenesis – Precursor of fragrance – Antifungal activity	– Reduced 13*S*-HODE level in colon and lung cancer cell/down regulate PPAR-δ mediated apoptosis	Shureiqi et al. (2003)
15-HETE 	– Inhibition of cancer cell proliferation, adhesion, and metastasis – Anti-inflammation – Human actin ligand	– PPAR-γ ligand – Reduced in lung cancer tissue – Precursor of lipoxin	Moreno (2009)
14*S*,21*R*-dihydroxy DHA 	– Wound healing and angiogenesis activity in diabetes – Remedies mesenchymal stem cell function	– Biosynthesized from DHA by lipoxygenase and P450	Tian et al. (2011)
9,12,13-TODE 	– Antifungal activity to phytopathogenic fungi – Adjuvant for influenza vaccine	– Self-defensive product in plant	Nagai et al. (2010)

(continued on next page)

Table 1 (*continued*)

HFA	Usage or status	Feature	Reference
Lipoxin A_4 	– Resolution of inflammation, anti-inflammation and anti-allergy – Inhibition of natural killer cell – Human protein kinase C activator	– Endogenous chemical resolving agent from AA – Act at subnanomolar concentration – Target receptors were identified	Chiang et al. (2006)
Resolvin E_1 	– Resolution of inflammation and reduce of inflammatory pain – Regulation of tissue homeostasis – Pre-clinical trials for asthma	– Biosynthesized from EPA – Target receptor was identified	El Kebir et al. (2012)

fatty acids and α,ω-dicarboxylic acid (Eschenfeldt et al., 2003), is high, whereas the activities of bacterial CYP105, 107, 109, and 153 are low (Fabritius et al., 1996). To date, most studies on ω-hydroxylases have been focused on CYP102 family enzymes. These enzymes are self-sufficient in their ability to transfer an electron from NADPH during its oxidation with a notably high turn-over number (>1000 min^{-1}) because they contain a flavin reductase as a redox-partner in a single protein (Fig. 2a). However, other CYP family ω-hydroxylases do not contain redox-partners. CYP102 family ω-hydroxylases show similar substrate specificity for C_{12}–C_{18} saturated fatty acids but remarkably different specificity for unsaturated fatty acids (Jung et al., 2011). CYP102A1, A2, A3, and D1 ω-hydroxylases prefer unsaturated fatty acids, including palmitoleic acid (16:1), linoleic acid, and arachidonic acid, whereas CYP102A7 ω-hydroxylase prefers saturated fatty acids. CYP102A1 ω-hydroxylase mainly catalyzes hydroxylation at the ω-2 position (80%) in arachidonic acid (Capdevila et al., 1996). However, CYP102A2 and CYP102A3 ω-hydroxylases catalyze hydroxylation at the evenly distributed positions from ω-1 to -3 (Gustafsson et al., 2004).

α-Hydroxylases catalyze hydroxylation at the α- and β-positions of the carboxyl end of saturated fatty acids (C_{12}–C_{18}) and unsaturated fatty acids, which are converted to 2-hydroxy and 3-hydroxy fatty acids, respectively, with high catalytic turn-over (>1000 min^{-1}). α-Hydroxylase activity is inhibited by catalase but increased by H_2O_2, with replacement of NADH and H_2O, as an oxygen donor (Matsunaga et al., 1996). CYP152 from *Sphingomonas paucimobilis* catalyzes hydroxylation only at the α-position of fatty acids (Matsunaga et al., 2000). However, CYP152 from *Bacillus subtilis* catalyzes hydroxylation at α-position (40%) and β-position (60%) in fatty acids (Matsunaga et al., 1999). Therefore, ω-hydroxylases and α-hydroxylases can be used as biocatalysts to produce ω-1, ω-2, and ω-3 hydroxy fatty acids and 2- and 3-hydroxy fatty acids, respectively.

3.2. Hydratases

Hydratases (EC 4.2.1.53) catalyze the regio-specific, irreversible addition of a hydrogen atom and a hydroxy group from water to the carbon–carbon *cis*-double bond of unsaturated fatty acids at the C9 and C10 positions, respectively, to make 10-hydroxy fatty acids (Table 2, Fig. 1b) (Joo et al., 2012a). Oleate hydratases convert oleic acid to 10-hydroxyoctadecanoic acid (10-hydroxystearic acid). Linoleate isomerases produce the intermediate 10-hydroxy-12(*Z*)-octadecenoic acid from linoleic acid during the formation of conjugated linoleic acid in *Lactobacillus* and *Bifidobacterium* (Ogawa et al., 2001; Rosberg-Cody et al., 2011). Linoleate isomerases and oleate hydratases differ at the genetic level.

Hydration reactions for unsaturated fatty acids have been found in many bacteria. *Stenotrophomonas nitritireducens* has the highest hydration activity toward linoleic acid at the C-10 position (Yu et al., 2008b), whereas other bacteria such as *Enterococcus gallinarum*, *Flavobacterium* sp., *Lactobacillus* sp., *Pediococcus acidilactici*, and *Selenomonas ruminantium*, have the highest activity toward oleic acid (Hou, 1994, 1995; Hudson et al., 1995, 1996; Morvan and Joblin, 1999). Oleate hydratases show the highest hydration activity for oleic acid (Joo et al., 2012a,b; Kim et al., 2012). These enzymes convert *cis*-9 and *cis*-12 unsaturated fatty acids into 2 products, either 10- or 13-hydroxy fatty acids (Joo et al., 2012a) and 13-hydroxy and 10,13-dihydroxy fatty acids (Volkov et al., 2010), and convert ricinoleic acid to 10,12-dihydroxystearic acid (Seo et al., 2013). Recently, oleate hydratases have been applied in a novel conversion process for the production of renewable ω-hydroxycarboxylic and α,ω-dicarboxylic acids (Song et al., 2013). Thus, hydratases can be used as biocatalysts to produce 10-hydroxy fatty acids and 10,13-dihydroxy fatty acids.

3.3. 12-Hydroxylases

12-Hydroxylases (EC 1.14.13.26) convert oleic acid to ricinoleic acid and catalyze the site-specific hydroxylation of the 12-position of oleic acid in the presence of NADH using O_2 (Table 2, Fig. 1c). The enzymes have been identified in the castor plants *Ricinus communis* (James et al., 1965), *Lesquerella fendleri* (Broun et al., 1998a), and *Lesquerella lindheimeri* (Dauk et al., 2007) and in the fungus *Claviceps purpurea*, a parasitic pathogen of cereal crops (Morris et al., 1966). The amino acid sequences of 12-hydroxylases are highly homologous with those of Δ12-desaturases, which convert oleic acid to linoleic acid by creating a double bond (van de Loo et al., 1995). Seven amino acid residues in 12-hydroxylase may determine the activity of 12-hydroxylases or Δ12-desaturases (Broadwater et al., 2002; Broun et al., 1998b). However, conclusive studies have not been reported. 12-Hydroxylase from *R. communis* converts oleic acid, linoleic acid, and 11-eicosenoic acid ($20{:}1^{\Delta 11Z}$) to ricinoleic acid, densipolic acid (12-hydroxyoctadec-9*Z*,15*Z*-dienoic acid, 12-OH $18{:}2^{\Delta 9Z,15Z}$), and lesquerolic acid (14*R*-hydroxyeicos-11*Z*-enoic acid, 14-OH $20{:}1^{\Delta 11Z}$), respectively (Broun and Somerville, 1997). 12-Hydroxylases from *L. fendleri* and *L. lindheimeri* convert oleic acid and palmitoleic acid ($16{:}1^{\Delta 9Z}$) to ricinoleic acid and 12-hydroxy palmitoleic acid, respectively (Broun et al., 1998a; Dauk et al., 2007).

Currently, mass production of ricinoleic acid from crops is limited because the castor plants produce the toxic and potentially lethal protein ricin (Knight, 1979). The fungus *C. purpurea* is an alternative source to accumulate a high level of ricinoleic acid (Morris et al., 1966).

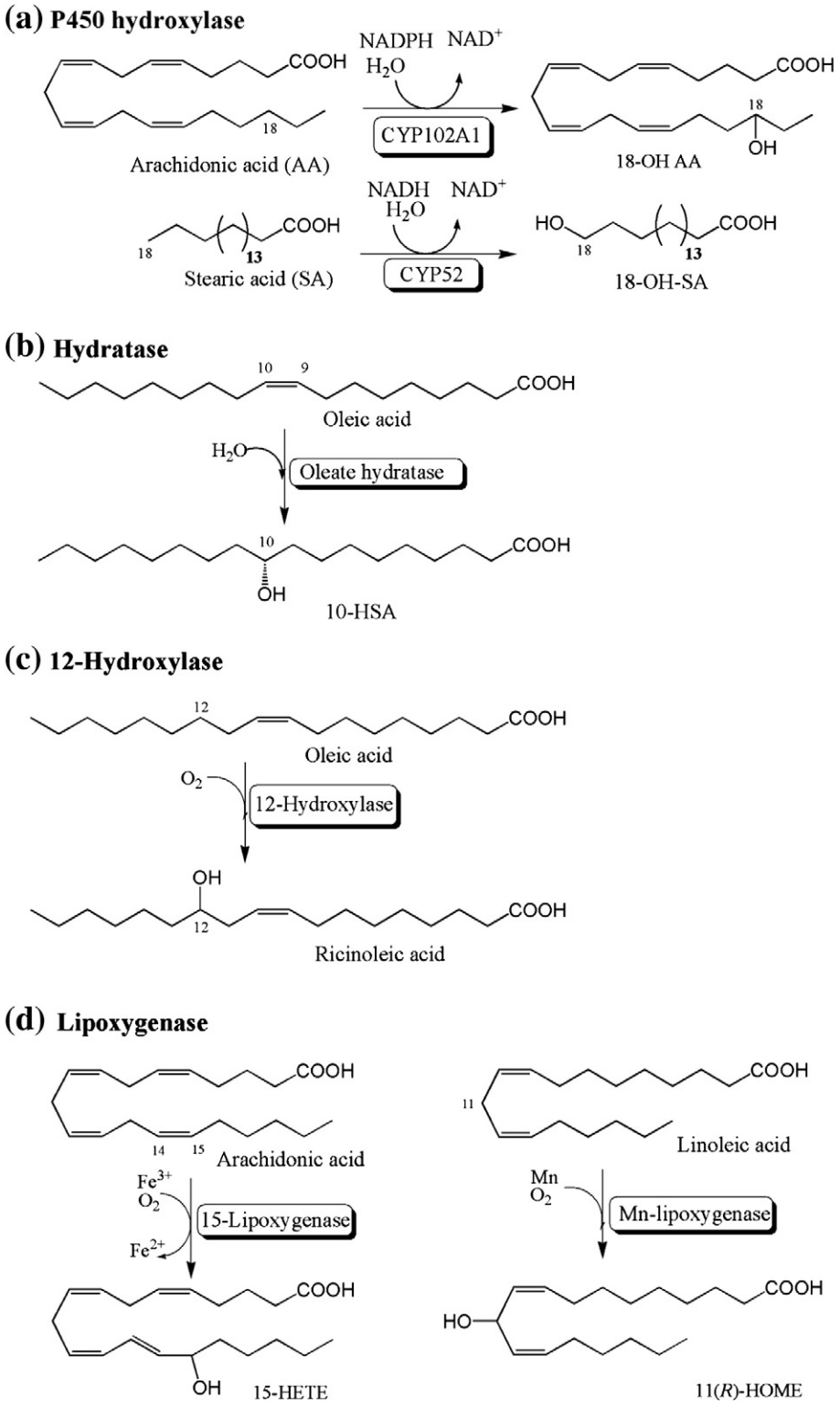


Fig. 1. Catalytic modes of fatty mono-hydroxylation enzymes.

However, commercialized production of ricinoleic acid is precluded because the fungus produces toxic ergot alkaloids (Mantle and Nisbet, 1976). The gene function and mechanism of the 12-hydroxylase from *C. purpurea* and those from the castor plants are almost identical (Billault et al., 2004; Holic et al., 2012; Meesapyodsuk and Qiu, 2008). Recombinant cells expressing the 12-hydroxylase gene of *C. purpurea* do not produce ergot alkaloids. Therefore, the recombinant cells are potential new biocatalysts for the production of ricinoleic acid.

3.4. Lipoxygenases

Lipoxygenases (EC 1.13.11), a family of dioxygenases, catalyze the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) containing one or more *cis,cis*-pentadiene units to produce the corresponding hydroperoxy fatty acids, which are reduced hydroxy fatty acids (Table 2, Fig. 1d) (Brash, 1999; Ivanov et al., 2010). Regiospecific lipoxygenases containing iron catalyze the positional-specific

Table 2
Summary of the enzymatic features of fatty acid hydroxylation enzymes.

Enzymes	EC number	OH-donor	OH-acceptor (metal)	Main substrate[a]	OH-position of products[b]	Structure[c]	Reference
P450							Lee et al. (2003)
α-hydroxylase	1.11.2.4	H_2O_2	Heme-iron	Alkane, AA	2-, 3-PA, 2-AA	3AWM, 1IZO	
ω-hydroxylase	1.14.15.3	H_2O	Heme-iron	Alkane, PUFA	19-, 18-AA	Unknown	
Unspecific	1.14.14.1	O_2	Heme-iron	AA, LA	18-,19-, 20-AA, LA	2HPD, 1FAG	
Hydratase	4.2.1.53	H_2O	Unknown	OA	10-HSA	Unknown	Bevers et al. (2009)
Lipoxygenase	1.13.11.-	O_2	Iron	AA	5-, 8-, 12-, 15-AA	3O8Y, 2FNQ,	Ivanov et al. (2010)
				LA	9-, 13-LA	3RDE, 1LOX, 1F8N, 1IK3	Hamberg et al. (1998)
			Manganese	LA	11-, 13-LA	Unknown	
12-Hydroxylase	1.14.13.26	O_2	Di-iron	OA	12-HOME (RA)	Unknown	van de Loo et al. (1995)
Diol synthase	1.13.11.44	O_2	Iron	LA	5,8-, 7,8-, 8,11-diHODE	Unknown	Jernerén et al. (2010a)

[a] Alkane, C_{12}–C_{18} saturated fatty acid; AA, arachidonic acid; LA, linoleic acid; OA, oleic acid; LnA, linolenic acid.
[b] PA, palmitic acid; HSA, hydroxystearic acid; RA, ricinoleic acid.
[c] Selected PDB accessions refined at high resolution (<2.5 Å).

hydroxylation of PUFAs (Joo and Oh, 2012). Animal 5-, 8-, 12-, and 15-lipoxygenases and microbial 15-lipoxygenase produce 5-, 8-, 12-, and 15-HETE from arachidonic acid. Lipoxygenases from human epidermal cells produce 7- and 9-HETE from arachidonic acid (Zheng and Brash, 2010), and 11-lipoxygenases from coral and sea urchin produce 11-HETE (Mortimer et al., 2006). DHA ($22{:}6^{\Delta 4Z,7Z,10Z,13Z,16Z,19Z}$) or EPA is also a substrate for producing 5-, 8-, 12-, and 15-hydroxy-DHA or hydroxy-EPA. Plant and bacterial 9- and 13-lipoxygenases and fungal 11- and 13-lipoxygenases produce 9-, 11-, and 13-HODE from linoleic acid. Fungal Mn-lipoxygenases produce 11- and 13-hydroxy octadecatrienoic acid (HOTE) from α-linolenic acid ($18{:}3^{\Delta 9Z,12Z,15Z}$). Leukotriene B_4, a 5,12-diHETE, is biosynthesized by the sequential cooperation of 5- and 12-lipoxygenases (Shimizu et al., 1984). The tri-hydroxy fatty acids lipoxin A_4 and B_4 are produced from arachidonic acid as a combinatory product of 5-, 12-, and 15-lipoxygenases (Serhan, 2002). Thus, lipoxygenases can be applied as biocatalysts to produce 5-, 7-, 8-, 9-, 11-, 12-, and 15-HETEs; 5-, 8-, 12-, and 15-HEPE; 5-, 8-, 12-, and 15-hydroxy-DHA; 9-, 11-, and 13-HODE; and 11- and 13-HOTE, as precursors of leukotriene and lipoxin signaling compounds.

4. Fatty acid di-hydroxylation enzymes

4.1. Bacterial diol synthases

The bacterial diol synthase from *Nostoc* sp. PCC 7120 is a fusion protein, consisting of lipoxygenase and allene oxide synthase (Lang et al., 2008) (Fig. 2a). The diol synthase converts linoleic acid to 9,14-diHODE as a major product with 9,10-, and 8,11-diHODE, and 9-HODE (Fig. 2b); α-linolenic acid to 9,16- and 9,13-diHOTE and 9-HOTE; γ-linolenic acid to 9,14-diHOTE and 9-HOTE; and arachidonic acid to 11,16-diHETE and 11-HETE. *Pseudomonas aeruginosa* 42A2 and PR3 strains produce 7,10-dihydroxy-8(*E*)-hexadecenoic acid (DHD) from palmitoleic acid (Bae et al., 2010), 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid (Gardner and Hou, 1999; Hou and Bagby, 1991; Hou et al., 1991), 7,10-diHODE from linoleic acid (Kuo and Nakamura, 2004), and 7,10,12-TODE from ricinoleic acid (Kuo et al., 2001). The hydroxylation reactions at C-7 and C-10 may be due to the conversions of P450-like isomerase and lipoxygenase, respectively (Kim et al., 2000a; Vidal-Mas et al., 2005). However, the hydroxylation at C-7 is not well understood because its different hydroxylation pattern differs from the positioning of typical dioxygenases.

Bacillus megaterium ALA2 and *Clavibacter* sp. ALA2 produce 12,13-diHODE from linoleic acid (Hou and Hosokawa, 2005; Hou et al., 1998). *Nostoc punctiforme* converts linoleic acid to 9- and 13-HODE (Lang and Feussner, 2007) and the green alga *Ulva conglobata* converts α-linolenic acid to 9- and 13-HOTE (Akakabe et al., 2002). However, these two organisms do not produce dihydroxy fatty acids. The red alga *Gracilariopsis lemaneiformis* converts arachidonic acid to 9,10- and 12,13-diHETE and 12-HETE (Gerwick et al., 1991; Hamberg and Gerwick, 1993). Therefore, diol synthases from bacteria and algae can be applied as biocatalysts in the production of 7,10-DHD; 6,9-, 7,10-, and 11,14-DOD; 7,10-, 7,12-, 8,11-, 9,10-, 9,14-, and 12,13-diHODE; 9,13-, 9,14-, and 9,16-diHOTE; and 11,16-diHETE.

4.2. Fungal diol synthases

Fungal diol synthases are fusion proteins, consisting of an N-terminal peroxidase (dioxygenase) domain and a C-terminal hydroperoxide isomerase/P450 domain (Fig. 2a). The enzymes catalyze the sequential dioxygenation of linoleic acid to dihydroxyoctadecadienoic acid (diHODE): 8-Hydroperoxy-(9*Z*,12*Z*)-octadecadienoic acid (HPODE) is first converted from linoleic acid by the diol synthase domain and subsequently isomerizes to 5,8-, 7,8-, and 8,11-diHODE by the P450 domain (Su et al., 1998) (Fig. 2c). Three diol synthases from fungi have been reported: PpoA [psi (precocious sexual inducer)-producing oxygenase A], PpoB, and PpoC from *Aspergillus nidulans* and *Aspergillus fumigatus*. PpoA is a 5,8-diol synthase that produces 8-HODE and 5,8-diHODE from linoleic acid (Brodhun et al., 2009). PpoB is an 8,11-diol synthase (Jernerén and Oliw, 2012). PpoC produces only 10-HODE from linoleic acid without forming dihydroxy fatty acid because the P450 domain of PpoC is non-functional (Brodhun et al., 2010). PpoA converts oleic acid to 8- and 10-hydroxyoctadecenoic acid (HOME) (Brodhun et al., 2009). PpoC also converts oleic acid, α-linolenic acid, and arachidonic acid to 10-HOME, 10-HOTE, and 10-, 11-, 12-, 13-, and 15-HETE, respectively (Garscha and Oliw, 2009).

The fungi *Laetisaria arvalis*, *Lasiodiplodia theobromae*, *Gaeumannomyces graminis*, *Magnaporthe grisea*, and *Magnaporthe oryzae* convert linoleic acid to 8-HODE, 9-HODE, and 7,8-diHODE (Bowers et al., 1986; Brodowsky et al., 1992; Cristea et al., 2003; Jernerén et al., 2010b). *Agaricus bisporus* converts linoleic acid to 8-HODE and 8,11-diHODE (Wadman et al., 2005). *Aspergillus* strains, including *Aspergillus clavatus*, *A. fumigates*, *A. nidulans*, and *A. niger*, convert linoleic acid to 5,8- and 8,11-diHODE (Jernerén et al., 2010a; Wadman et al., 2009). *Leptomitus lacteus* converts linoleic acid to 7- and 8-HODE and to 8,11-, 11,16-, and 11,17-diHODE (Fox et al., 2000). The diol synthase genes from these fungi are new sources for the production of novel di-hydroxy fatty acids. Thus, diol synthases from these fungi can be utilized as biocatalysts in the production of not only 5,8-, 7,8-, 8,11-, 11,16-, and 11,17-diHODE but also 7-, 8-, 9-, and 10-HODE, 10-HOME, 10-HOTE, and 10-, 11-, 12-, 13-, and 15-HETE.

5. Production of hydroxy fatty acids

The diverse kinds of hydroxy fatty acids are produced using wild-type and recombinant whole cell conversion, fermentation, and enzymatic

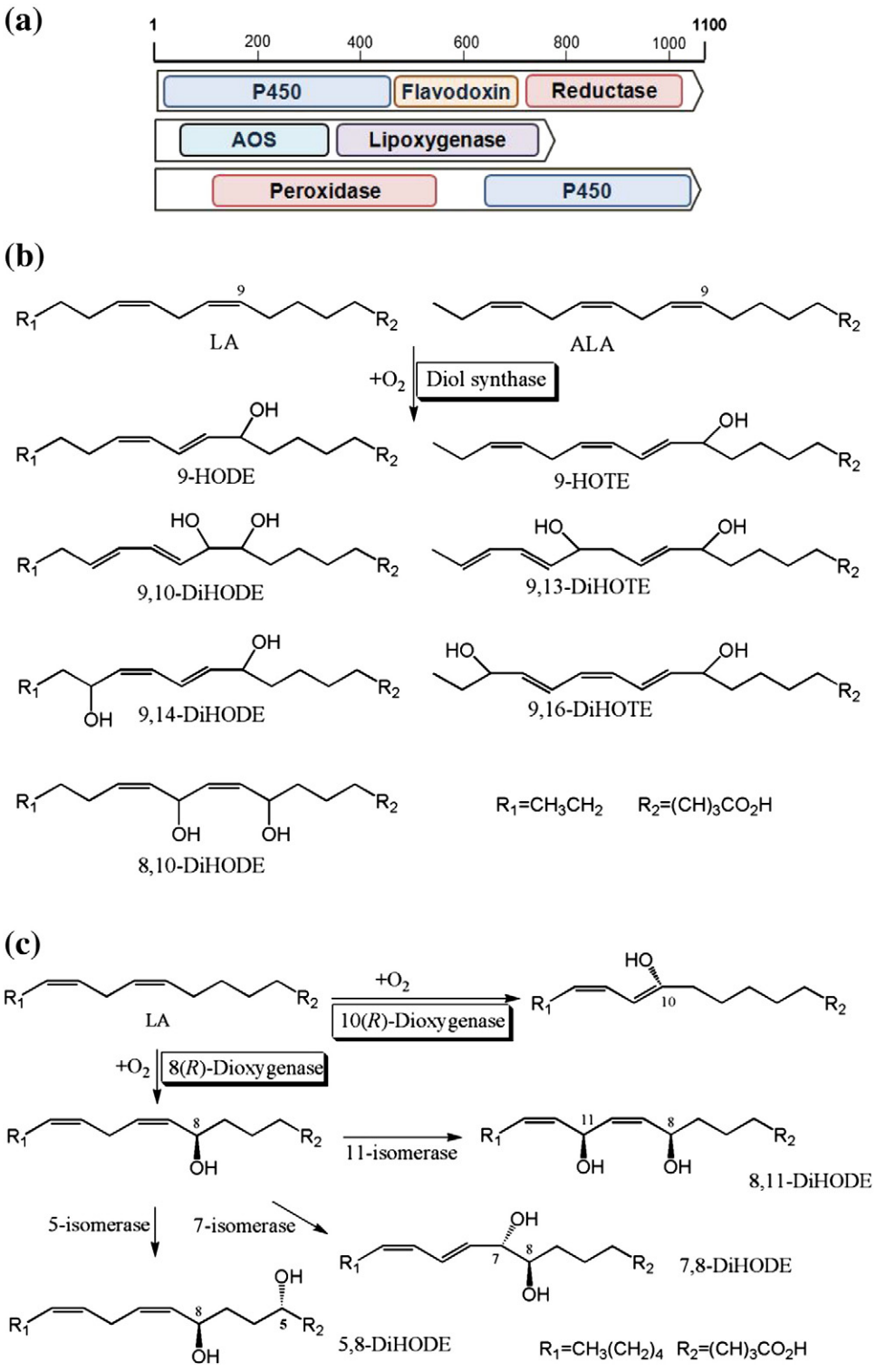


Fig. 2. (a) Structure of natural fusion proteins. Redox-partner containing P450 CYP102 family enzymes (upper), diol synthases from bacteria (middle), and diol synthases from fungi (lower). The numbers of the amino acid residues are presented on the line. (b) Biosynthesis of di-hydroxy fatty acids by diol synthases from bacteria. (c) Biosynthesis of di-hydroxy fatty acids by diol synthases from fungi.

conversion (Table 3). Many studies describe the production of 10-hydroxystearic acid by whole cell conversions using wild-type (Hudson et al., 1995; Kuo et al., 2000; Morvan and Joblin, 1999) and recombinant microorganisms (Kim et al., 2012). Whole cells of recombinant *Escherichia coli* expressing the oleate hydratase gene of *Stenotrophomonas maltophilia* produce 10-hydroxystearic acid from oleic acid with the highest concentration and productivity of (Joo et al., 2012b). *P. aeruginosa* strains produce mono- and di-hydroxy fatty acids by cell conversions (Bae et al., 2010; Chang et al., 2008; Culleré et al., 2001; Suh et al., 2011), *B. megaterium* ALA2 strains produce tri-hydroxy fatty acids (Kim et al., 2010), and recombinant *Schizosaccharomyces pombe* expressing the oleate 12-hydroxylase gene of *C. purpurea* produce ricinoleic acid from oleic acid with an overall yield of 52.6% (Holic et al., 2012). Recombinant *E. coli* expressing the CYP505 gene of *Fusarium oxysporum* produces mostly ω-1 positioned hydroxy fatty acids from palmitic acid, linoleic acid, and waste cooking

Table 3
Production of hydroxy fatty acids by wild-type and recombinant whole cell conversion, fermentation, and enzymatic conversion.

Microorganism	Substrate[a] ($g\ l^{-1}$)	Product[b] ($g\ l^{-1}$)	Y ($g\ g^{-1}$)	Q_p ($g\ l^{-1}\ h^{-1}$)	qP ($g\ g\text{-}cell^{-1}\ h^{-1}$)	Method	Reference
Bacillus megaterium ALA2	LA (3.6)	12,13,17-TODE (0.65)	0.18	0.013	ND[c]	Cell conversion	Kim et al. (2010)
Candida tropicalis	OA(43.0)	3-HOME (19.4)	0.45	0.087	ND	Cell conversion	Fabritius et al. (1996)
Clavibacter sp. ALA2	ALA (2.5)	DHTHF(0.9)	0.36	ND	ND	Cell conversion	Hosokawa et al. (2003)
Enterococcus faecalis	OA(0.9)	10-HSA (0.80)	0.89	ND	ND	Cell conversion	Hudson et al. (1995)
	LA(1.0)	10-ODA[c] (0.22)	0.22	ND	ND	Cell conversion	Hudson et al. (1998)
E. gallinarum	OA (2.0)	10-HSA (1.9)	0.95	0.027	ND	Cell conversion	Morvan and Joblin (1999)
E. coli containing oleate hydratase from *S. maltophilia*	OA (50.0)	10-HSA (49.0)	0.98	12.6	1.23	Cell conversion	Joo et al. (2012b)
	OA (50.0)	10-HSA (45.5)	0.91	8.2	2.0	Fermentation	Jeon et al. (2012)
E. coli containing CYP505 Foxy	PA (0.77)	1-HPA (0.018)	0.02	ND	ND	Cell conversion	Kitazume et al. (2008)
	LA (0.6)	1-OH LA (0.14)	0.23	ND	ND		
	WL (22.0)	1-HPA (0.012)	0.001	ND	ND		
Flavobacterium sp. DS5	OL (18.0)	10-HSA (0.71)	0.04	0.015	ND	Cell conversion	Heo et al. (2009)
	OA (5.40)	10-HSA (2.97)	0.55	0.083	ND		
Lactobacillus sp.	OA (2.0)	10-HSA (1.5)	0.76	0.011	ND	Cell conversion	Morvan and Joblin (1999)
L. acidophilus	OA (5.0)	10-HSA (1.45)	0.29	0.030	ND	Cell conversion	Ogawa et al. (2001)
L. paracasei	OA (2.0)	10-HSA (1.82)	0.91	0.011	ND	Cell conversion	Kishimoto et al. (2003)
Lysinibacillus fusiformis	OA (40.0)	10-HSA (40)	1.0	0.011	ND	Enzymatic conversion	Kim et al. (2012)
	OL (40.0)	10-HSA (40)	1.0	0.01			
Nocardia cholesterolicum	SL (17.8)	10-HSA (16.0)	0.90	2.7	0.03	Cell conversion	Koritala and Bagby (1992)
	OA (17.5)	10-HSA (12.4)	0.71	0.518	0.015	Cell conversion	Koritala et al. (1989)
N. paraffinae	OA (18.0)	10-HSA (8.0)	0.44	1.6	0.46	Cell conversion	Latrasse et al. (1997)
Pediococcus acidilactici	OA (2.0)	10-HSA (1.9)	0.93	0.013	ND	Cell conversion	Morvan and Joblin (1999)
Pseudononas aeruginosa 42A2	OA (14.0)	10-ODA (6.77)	0.48	0.14	ND	Immobilization	Culleré et al. (2001)
	WFO (20.0)	10-ODA (7.5)	8.7	0.46	0.46	Fermentation	Torrego-Solana et al. (2012)
		DOD (13.4)	12.8	0.41	0.41		
P. aeruginosa PR3	EA (6)	DED (0.21)	0.37	ND	ND	Cell conversion	Back et al. (2011)
	OL (10)	DOD (4)	0.4	ND	ND	Cell conversion	Suh et al. (2011)
	PTA (6)	DHD (1.6)	0.27	ND	ND	Cell conversion	Bae et al. (2010)
	TOL (6)	DOD (1.88)	0.31	ND	ND	Cell conversion	Chang et al. (2008)
	OA (22.2)	DOD (8.27)	0.37	ND	ND	Cell conversion	Kuo and Lanser (2003)
Schizosaccharomyces pombe containing *CpFAH12*	OA (0.07)	RA (0.03)	0.42	ND	ND	Cell conversion	Holic et al. (2012)
Selenomonas ruminantium	OA (0.9)	10-HSA (0.63)	0.70	0.009	ND	Cell conversion	Hudson et al. (1995)
Sphingobacterium thalpophilum	SL (18.0)	10-HSA (7.1)	0.39	0.074	ND	Cell conversion	Kuo and Levinson (2006)
Stenotrophmonas maltophilia	OA (50.0)	10-HSA (40.0)	0.80	5.0	0.50	Cell conversion	Joo et al. (2012b)
S. nitritireducens	OA (30.0)	10-HSA (31.5)	1.05	7.9	0.39	Cell conversion	Kim et al. (2011)
	LA (20.0)	10-ODA (14.9)	0.75	7.50	0.375	Cell conversion	Yu et al. (2008a)

[a] OA, oleic acid; OL, olive oil; LA, linoleic acid; ALA, α-linolenic acid; EA, eicosenoic acid; PA, palmitic acid; PTA, palmitoleic acid; SL, soybean oil; TOL, triolein; WFO, waste frying oil.
[b] 10-HSA, 10-hydroxystearic acid; 10-ODA, 10-hydroxy-8-octadecenoic acid; DED, 9,12-dihydroxy-10-eicosenoic acid; RA, ricinoleic acid; DHTHA, dihydroxy-tetrahydroxyfuranyl fatty acids; HPA, hydroxypalmitic acid.
[c] ND: Not determined.

oil (Kitazume et al., 2008). Hydroperoxy fatty acids as precursors of hydroxy fatty acids have been produced from unsaturated fatty acids by lipoxygenases. Soybean 13-lipoxygenases and fungal Mn-lipoxygenase produce 13-HPODE from linoleic acid (Drouet et al., 1994; Iacazio et al., 1990; Villaverde et al., 2013).

Substrates used to produce hydroxy fatty acids include not only saturated and unsaturated free fatty acids, such as palmitic acid, palmitoleic acid, oleic acid, linoleic acid, and α-linolenic acid, but also oil hydrolyzates, which are hydrolyzed to fatty acids from olive, soybean, and waste oils by lipase or hydrolysis methods (Heo et al., 2009; Kim et al., 2012). The use of surplus oils, such as soybean oil, is a desirable way to produce hydroxy fatty acids because such oils are inexpensive resources (Bagby and Carlson, 1989; Hou and Hosokawa, 2005). The reaction conditions, such as pH, temperature, oxygen supply, and the addition of surfactants and organic solvents, have been optimized for the production of hydroxy fatty acids. Fatty acid-hydroxylation enzymes except for oleate hydratase require oxygen as a hydroxylation donor for catalysis. Thus, agitation is necessary for oxygen or air supplementation. The optimization of pH and temperature increases the production of hydroxy fatty acids (Bae et al., 2010; Chang et al., 2008; Kim et al., 2012). Surfactants and organic solvents are effective to increase the production of hydroxy fatty acids because these agents act as emulsifiers to help dispersion and mixing and as assistants to expose the hydrophobic substrate to enzymes or cells (Kim et al., 2010, 2012).

6. Perspectives

6.1. Gene screening

The genes encoding fatty acid-hydroxylation enzymes from animals, plants, and microorganisms have been characterized, and the enzymes have been used as biocatalysts to produce hydroxy fatty acids (Senger et al., 2005; Tsitsigiannis et al., 2005; Zheng and Brash, 2010). Although approximately 20,000 genome sequences have been identified to data, many genes encoding fatty acid-hydroxylation enzymes remain uncharacterized. Among them, genes from microorganisms, such as oleaginous microorganisms (Huang et al., 2013), can be screened more easily from environmental samples using a high-throughput screening (HTS) method (Schwaneberg et al., 2001; Whent et al., 2010) than those from other organisms. Moreover, microorganisms produce more regio-specific and diverse hydroxy fatty acids.

Sequence-based screening of metagenome libraries is a potentially useful approach for the obtainment of new genes encoding fatty acid-hydroxylation enzymes (Kim et al., 2007). This screening method obtains genes directly from environmental samples without cultivation, and genes of uncultured organisms are also obtained. The codons of the obtained genes are optimized for easy expression in a microbial host, and codon-optimized synthetic genes are potential sources for the production of novel hydroxy fatty acids. Thus, gene

screening of microbial sources from metagenome libraries and environmental samples is a useful way to find novel and diverse hydroxy fatty acids.

6.2. Protein engineering

Protein engineering has emerged as a powerful technology for the development of useful or valuable biocatalysts. The protein engineering of fatty acid-hydroxylation enzymes, including P450 and lipoxygenase, has been used to alter regio-specificity (Chen et al., 2008; Dietrich et al., 2009), substrate specificity (Axarli et al., 2005), metal selectivity (Wennman et al., 2012), and thermostability (Mandai et al., 2009). Many studies have focused on altering the regio-specificity of lipoxygenases. The replacement of amino acid residues in lipoxygenase alters the C9 and C13 oxygenation positions in linoleic acid (Hornung et al., 1999). The oxygenation positions in arachidonic acid have been changed from C5 to C15 (Schwarz et al., 2001), C8 to C12 (Coffa and Brash, 2004) or C15 (Jisaka et al., 2000), C12 to C8 or C11 (Meruvu et al., 2005), and C15 to C12 (Sloane et al., 1995) by replacing amino acid residues in lipoxygenases. To produce more diverse hydroxy fatty acids, the regio-specificity of P450, hydratase, 12-hydroxylase, and dioxygenase should be altered by protein engineering.

6.3. Metabolic engineering

The biosynthesis of hydroxy fatty acids is associated with the β-oxidation metabolism of fatty acids. Intermediates in the β-oxidation pathway are used as substrates in the intracellular synthesis of polyhydroxyalkanoic acid (PHA), which accumulates in bacterial cells for energy storage. A knock-out mutant deficient in the synthesis of PHA produces the extracellular product 10-HODE from oleic acid with an enhanced carbon uptake rate (Torrego-Solana et al., 2012). To increase the production of hydroxy fatty acids, genes involved in the production of nonessential compounds from the β-oxidation metabolism of fatty acids must be deleted. Recently, a pathway was

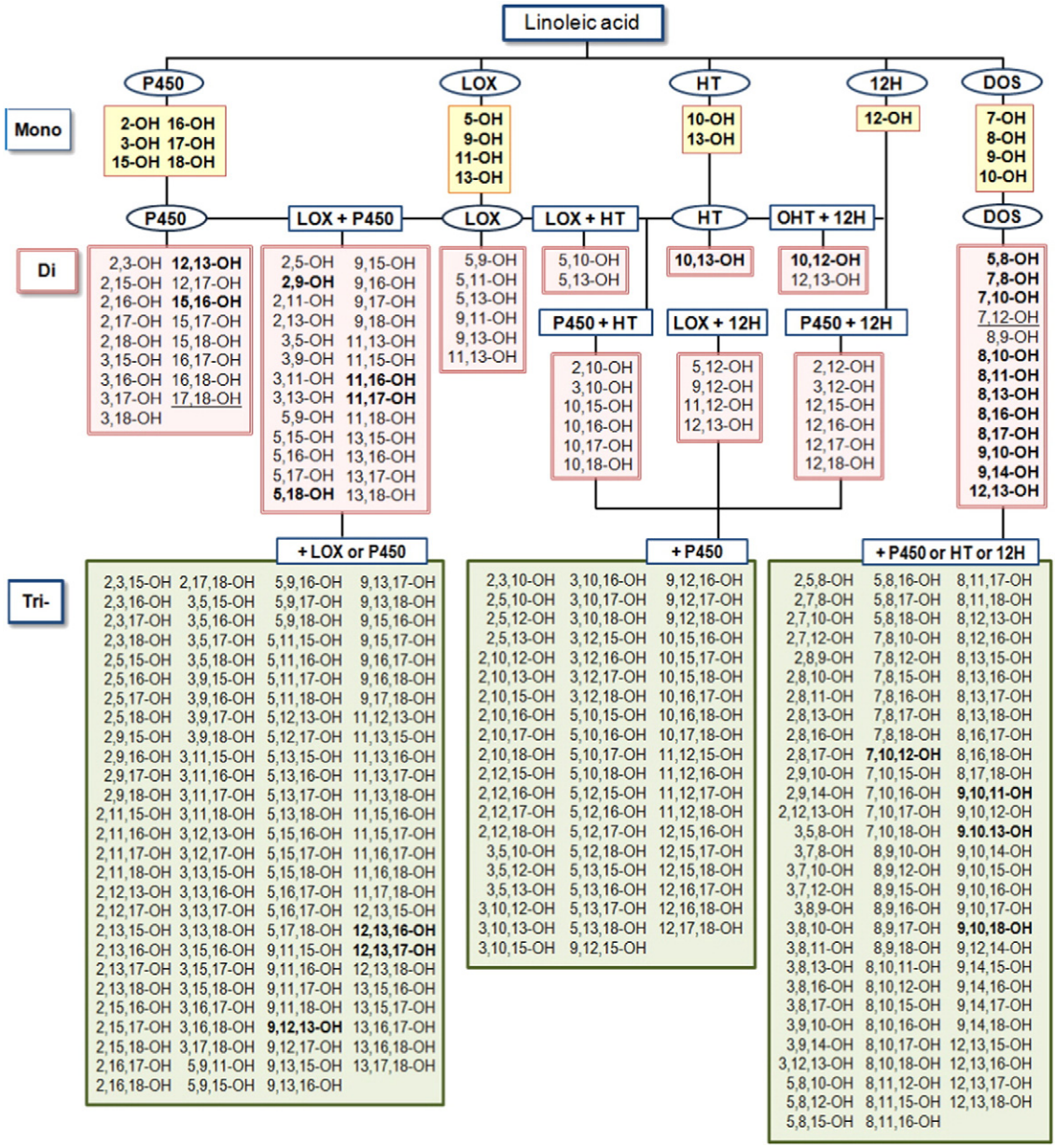


Fig. 3. Proposed diversification routes from combinatory biosynthesis by fatty acid-hydroxylation enzymes using linoleic acid. LOX: lipoxygenase, HT: hydratase, 12H: 12-hydroxylase, DOS: diol synthase. Bold and underlined letters indicate the reported and estimated hydroxy fatty acids, respectively.

engineered in *E. coli* to produce long chain hydroxy fatty acids directly from glucose (Wang et al., 2012). The fatty acid acyl-CoA synthase gene in *E. coli* was knocked out to block fatty acid degradation. Fatty acid thioesterase and P450 ω-hydroxylase are needed to produce free fatty acids and hydroxy fatty acids at the ω-1, ω-2, and ω-3 positions, respectively. *E. coli* engineered to express fatty acid thioesterase and P450 ω-hydroxylase genes without fatty acid acyl-CoA synthase gene accumulates ω-1, ω-2, and ω-3 positioned at C12- and C14-hydroxy fatty acids from glucose. Therefore, metabolic engineering is a practical tool for the increased and economic production of hydroxy fatty acids.

6.4. Combinatory biosynthesis

Di- and tri-hydroxy fatty acids can be synthesized from fatty acids via mono-hydroxy fatty acids by the combinatory use of fatty acid-hydroxylation enzymes. As an example, the biosynthesis of mono-, di-, and tri-hydroxy fatty acids from linoleic acid is shown in Fig. 3. Fourteen types of mono-hydroxy fatty acids have been produced from linoleic acid: 2-, 3-, 15-, 16-, 17-, and 18-hydroxy fatty acids by P450s (Boddupalli et al., 1992; Budde et al., 2004; Dietrich et al., 2009; Hamberg et al., 2003; Kandel et al., 2006; Matsunaga et al., 1998; Nakayama et al., 1996), 5-, 9-, 11-, and 13-hydroxy fatty acids by lipoxygenases (Joo and Oh, 2012), and 10- and 13-hydroxy fatty acids by hydratase (Joo et al., 2012a), 12-hydroxy fatty acid from linoleic acid by 12-hydroxylase (Meesapyodsuk and Qiu, 2008), and 7-, 8-, 9-, and 10-hydroxy fatty acids by the peroxygenase activity of diol synthases (Brodhun et al., 2009, 2010; Garscha and Oliw, 2009; Hoffmann et al., 2011; Jernerén and Oliw, 2012; Jernerén et al., 2012).

Sixteen types of di-hydroxy fatty acids have been produced from linoleic acid: 2,9-diHODE by tobacco α-hydroxylase and lipoxygenase (Hamberg et al., 2003), 8,13-diHODE via 8-HODE as an intermediate by lipoxygenase (Garscha and Oliw, 2007), 5,8-, 7,8-, and 8,11-diHODE by fungal diol synthases (Brodhun et al., 2009; Su et al., 1998), 7,10-, 7,12-, 8,11-, 9,10-, 9,14-, and 12,13-diHODE by bacterial diol synthases (Kuo and Nakamura, 2004; Lang et al., 2008), 9,10- and 12,13-diHODE via epoxy intermediates by plant epoxy-forming diol synthase-like peroxygenases (Hamberg and Hamberg, 1996) 9,10-, 12,13-, and 15,16-diHODE via epoxy intermediates by animal epoxy-forming P450s (Niehaus et al., 1970; Ruettinger and Fulco, 1981), 8,9-, 13,14-, and 17,18-diHODE by the fungus *G. lemaneiformis* (Gerwick et al., 1991), and 8,11-, 11,16-, and 11,17-diHODE by the fungi *L. lacteus* (Fox et al., 2000) and *M. grisea* (Cristea et al., 2003). Approximately 80 types of di-hydroxy fatty acids, including the reported di-hydroxy fatty acids, can theoretically be formed by the combinatory use of the same or different enzymes, including P450, lipoxygenase, hydratase, 12-hydroxylase, and diol synthase. The production of di-hydroxy fatty acids from unsaturated fatty acids containing *trans*-double bond by hydratases is excluded because hydratases have no activity for the *trans*-double bond. For example, the production of 10,13-di-hydroxy fatty acids from 13-hydroxy fatty acids by hydratase is not possible.

Seven types of tri-hydroxy fatty acids have been produced from linoleic acid: 9,10,11-TODE by *Tuber indicum* and *Solanum tuberosum* (Gao et al., 2001), 9,10,13- and 9,12,13-TODE by *S. tuberosum* (Hamberg, 1999), *Saprolegnia parasitica* (Hamberg, 1986), and *P. aeruginosa* PR3 (Kim et al., 2000b), 12,13,16- and 12,13,17-TODE by *B. megaterium* ALA2 and *Clavibacter* sp. ALA2 (Gardner et al., 2000; Hou, 1996, 1998, 2001, 2006), 9,12,13-TODE and 9,12,13-tri-HODE by lipoxygenase and P450 (Hamberg and Olsson, 2011), and 9,10,18-TODE by the cutin component of many plants (Lequeu et al., 2003). Over 200 types of tri-hydroxy fatty acids, including the reported tri-hydroxy fatty acids, can be proposed based on the combinatory biosynthesis by fatty acid-hydroxylation enzymes. The combinatory biosynthesis by fatty acid-hydroxylation enzymes is also applied to other unsaturated fatty acids, such as oleic acid, α-linolenic acid, γ-linolenic acid, and arachidonic acid. Therefore, the combinatory use of fatty acid-hydroxylation enzymes is a practical and easy method to produce diverse hydroxy fatty acids.

7. Conclusion

Hydroxy fatty acids are important materials in industry and medicine. The research and demand in chemical, agricultural, food, cosmetic, and pharmaceutical industries are steadily increasing, but mass production and diversification remain limited. To produce diverse hydroxy fatty acids, gene screening, protein engineering, metabolic engineering, and the combinatory use of fatty acid-hydroxylation enzymes are essential. Inexpensive resources and appropriate biocatalysts and hosts are useful for the industrial production of hydroxy fatty acids. The biological activities and properties of hydroxy fatty acids should also be investigated to find industrial and medicinal applications.

Abbreviations

(P450) Cytochrome P450 monooxygenase
(DHD) dihydroxyhexadecenoic acid
(DOD) dihydroxyoctadecenoic acid
(DHA) docosahexanoic acid
(EPA) eicosapentaenoic acid
(HPODE) hydroperoxyoctadecadienoic acid
(HDHA) hydroxydocosahexaenoic acid
(HEPE) hydroxyeicosapentaenoic acid
(HETE) hydroxyeicosatetraenoic acid
(HODE) hydroxyoctadecadienoic acid
(HOME) hydroxyoctadecenoic acid
(HOTE) hydroxyoctadecatrienoate
(PHA) polyhydroxyalkanoic acid
(PUFA) polyunsaturated fatty acid
(TODE) trihydroxyoctadecenoic acid

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