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ω3 fatty acid desaturases from microorganisms: structure, function, evolution, and biotechnological use

Mingxuan Wang
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Haiqin Chen
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Ave, Wuxi, Jiangsu 214122, People's Republic of China

Zhennan Gu
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Ave, Wuxi, Jiangsu 214122, People's Republic of China

Hao Zhang
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Ave, Wuxi, Jiangsu 214122, People's Republic of China

Wei Chen
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Ave, Wuxi, Jiangsu 214122, People's Republic of China

Yong Q. Chen
State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, People's Republic of China

Synergetic Innovation Center of Food Safety and Nutrition, 1800 Lihu Ave, Wuxi, Jiangsu 214122, People's Republic of China

Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, NC 27157, USA

Abstract

The biosynthesis of very-long-chain polyunsaturated fatty acids involves an alternating process of fatty acid desaturation and elongation catalyzed by complex series of enzymes. ω3 desaturase plays an important role in converting ω6 fatty acids into ω3 fatty acids. Genes for this desaturase

haiqinchen@jiangnan.edu.cn.

have been identified and characterized in a wide range of microorganisms, including cyanobacteria, yeasts, molds, and microalgae. Like all fatty acid desaturases, ω3 desaturase is structurally characterized by the presence of three highly conserved histidine-rich motifs; however, unlike some desaturases, it lacks a cytochrome b5-like domain. Understanding the structure, function, and evolution of ω3 desaturases, particularly their substrate specificities in the biosynthesis of very-long-chain polyunsaturated fatty acids, lays the foundation for potential production of various ω3 fatty acids in transgenic microorganisms.

Keywords

Polyunsaturated fatty acids (PUFAs); ω3 desaturase; Lipid biochemistry; Substrate specificity; Biotechnology

Introduction

During the past few decades, very-long-chain polyunsaturated fatty acids (VLC-PUFAs) such as arachidonic acid (ARA; 20:4ω6), eicosapentaenoic acid (EPA; 20:5ω3), and docosahexaenoic acid (DHA; 22:6ω3) have attracted the attention of the scientific community as well as the dietary supplement and food industries due to their proven health benefits (Chen et al. 2006; Connor et al. 2007; Vanden Heuvel 2012). VLC-PUFAs play important roles as structural components of membrane phospholipids and as precursors of the eicosanoid family of signaling molecules, including prostaglandins, thromboxanes, and leukotrienes (Zhang et al. 2011). They not only exhibit various regulatory effects and physiological activities, but also play important roles in infant nutrition (Carlson et al. 1993; Gill and Valivety 1997).

Fish and fish oils are the major dietary sources of VLC-PUFAs, but concerns over the ongoing depletion of wild fish stocks and contamination of the oceans have led to the obvious conclusion that alternative sustainable sources are urgently needed (Racine and Deckelbaum 2007). Microorganisms are very promising lipid sources: they are a diverse group of organisms with important biochemical functions, have extremely high growth rates in simple media and can be manipulated easily. Some yeasts and molds are known as oleaginous microorganisms, accumulating high levels of triacylglycerols that sometimes reach and exceed 70 % of the biomass weight (Ratledge and Wynn 2002).

Fatty acid desaturases are enzymes responsible for the production of unsaturated (e.g. oleic acid) and polyunsaturated fatty acids (e.g. linoleic acid) (Fig. 1). Each fatty acid desaturase introduces a double bond at a specific position in the acyl chain (Los and Murata 1998). Except for Δ9 desaturases, which introduce the first double bond into saturated fatty acids, desaturases can be referred to as front-end desaturases or methyl-end desaturases based on the position of the double bond insertion relative to a pre-existing double bond in a fatty acyl chain (Aitzetmüller and Tsevegsüren 1994). ω3 desaturases are essential for the desaturation of ω6 fatty acids into ω3 fatty acids and belong to the class of methyl-end desaturases.

Several reviews have focused on studies of different types of desaturases (Meesapyodsuk and Qiu 2012; Pereira et al. 2003; Tocher et al. 1998), however, there is no review regarding ω3 desaturases. The genes coding for ω3 desaturases have been identified and characterized in a wide range of microorganisms, including cyanobacteria, yeast, mold, and microalgae. These microbial ω3 desaturases perform important function converting ω6 fatty acids into ω3 fatty acids with high activity and broad substrate specificities. Thus, it is necessary to timely summarize recent progresses, and we hope this inspires more research interests and advances related studies in this field.

In this review, we first summarize the advances in the biochemical characterization of various microbial ω3 desaturases and analyze the evolution of ω3 desaturases. Next, we discuss structural determinants for substrate specificity of ω3 desaturases. Finally, we highlight the importance of fungal ω3 desaturases in biotechnological use and describe its future perspective.

ω3 desaturases from different species

The first ω3 desaturase gene from a microorganism was cloned in 1994 from the cyanobacterium *Synechocystis* sp. PCC 6803; its histidine residues are well conserved, even though the amino acid sequence similarity between the cyanobacteria and higher-plant ω3 desaturases is not significant (Sakamoto et al. 1994). The first eukaryotic and fungal ω3 desaturase has been reported in 1997, when its gene was cloned from yeast *Saccharomyces kluyveri* (Oura and Kajiwara 2004). The predicted protein consists of 419 amino acids, and shows 29–31% identity with ω3 fatty acid desaturases from animals and plants (Oura and Kajiwara 2004). The ω3 desaturase gene from *Mortierella alpina* 1S-4 was cloned based on the conserved sequence information for *S. kluyveri* ω3 desaturase and *M. alpina* 1S-4 Δ12 desaturase. Homology analysis of protein databases revealed that the amino acid sequence of *M. alpina* ω3 desaturase was most closely related to *M. alpina* 1S-4 Δ12 desaturase (51 % identity), whereas it exhibited 36 % identity with *S. kluyveri* ω3 desaturase (Sakuradani et al. 2005). Another yeast ω3 desaturase was isolated from methylotrophic yeast *Pichia pastoris* GS115 (Zhang et al. 2008). The deduced amino acid sequence of this cloned cDNA showed high identity to known fungal ω3 fatty acid desaturases (Zhang et al. 2008). Besides these fungal ω3 desaturases, bifunctional Δ12/ω3 desaturases were recently identified from filamentous fungi *Fusarium moniliforme* (Damude et al. 2006) and two thermophilic fungi, *Myceliophthora thermophila* and *Thielavia terrestris* (Berka et al. 2011).

The function of ω3 desaturases isolated from prokaryotes and eukaryotes was mostly established by their expression in *S. cerevisiae* (Tocher et al. 1998). In the common biosynthesis pathway of very-long-chain polyunsaturated fatty acids, this desaturase typically uses 18-carbon and 20-carbon fatty acids as substrates. However, functional enzymatic studies have showed that ω3 desaturases isolated from different species have distinct substrate preferences. This will be discussed later.

Primary structure and evolution of ω3 desaturases

Based on solubility, desaturases are classified in two types: soluble and membrane-bound. ω3 desaturases are membrane-bound desaturases and they are distinguished from the front-end desaturases and Δ9 desaturases by the absence of an N-terminal fused cytochrome b5 domain (Pereira et al. 2003). Most front-end desaturases are fusion proteins with a cytochrome b5-like domain at the N-terminus, whereas Δ9 desaturases have the cytochrome b5-like domain in the C-terminus. Since the cytochrome b5-like domain is an essential part of these enzymes (Mitchell and Martin 1995; Qiu et al. 2002), ω3 desaturases may use free cytochrome b5 as an electron donor.

Examination of predicted amino acid sequences for the membrane desaturases from mammals, fungi, insects, higher plants, and cyanobacteria has revealed three conserved histidine-box motifs containing eight histidine residues with the general structure $HX_{(3\text{ or }4)}H$, $HX_{(2\text{ or }3)}HH$, and $HX_{(2\text{ or }3)}HH$ (Shanklin et al. 1994). ω3 desaturases are no exception, sharing three typical histidine-rich motifs that are likely involved in the catalysis of the desaturation reaction. Moreover, comparison of the three histidine boxes of ω3 desaturases from different organisms revealed that several residues within these motifs are highly conserved (Table 1), possibly reflecting the evolutionary relationship between the various species.

The evolution of membrane-bound desaturases as a whole has been reviewed by Alonso in 2003, but many important fungal ω3 desaturase genes, which were mostly characterized after 2003, were not included (López Alonso et al. 2003).

As shown in Fig. 2, the phylogenetic relationships between ω3 and Δ12 membrane desaturase genes from various organisms were analyzed using the neighbor-joining (NJ) method. As previously reported, three different lineages can be distinguished in the Δ12/ω3 desaturase group, and the most basal group is comprised of the cyanobacteria Δ12 and plant chloroplastic Δ12 desaturases. This suggests that Δ12 desaturases are ancestral to the ω3 desaturases (López Alonso et al. 2003). The second cluster group consists of ω3 desaturases from cyanobacteria and microalgae as well as microsomal and chloroplastic ω3 desaturases from higher plants. Their close similarity suggests that ω3 desaturases in the plant and microalgae endoplasmic reticulum (ER) are derived from a cyanobacteria ω3 desaturase source, most likely from a symbiont. Interestingly, a fungal ω3 desaturase gene from *Saprolegnia diclina* segregated with the second cluster, although bootstrap values at this branch point are low. The formation of the separate branch indicates that the ω3 desaturase gene from the EPA-rich fungus *S. diclina* evolved independently from other fungi. It is worthwhile to mention that the function of *S. diclina* ω3 desaturase is quite different from other fungal ω3 desaturases, as it exclusively desaturates 20-carbon ω6 fatty acids (Pereira et al. 2004).

Unlike the phylogenetic tree presented by Alonso, our analysis shows a third group comporting a mixture of Δ12 and ω3 desaturases. The presence of ω3 desaturase genes suggests that fungal ω3 desaturases arose by independent gene duplication events at different times from a Δ12 desaturase ancestor. The close similarity between fungal Δ12 and ω3 desaturases during evolution is also consistent with their similarity in primary structure. The ω3 desaturase gene of *Caenorhabditis elegans* represents the only representative of this class described in animals and it seems to be evolving in a different way.

From a functional point of view, microorganisms respond to a decrease in temperature by desaturating fatty acids in membrane lipids to compensate for the decrease in membrane fluidity. In cyanobacteria, which were used as a model system for studying sensitivity to chilling (Wada et al. 1990), the desaturation of fatty acids at the ω3 position is the most sensitive to the change in temperature of all desaturation reactions (Sakamoto et al. 1994). In evolutionary terms, the reason for the appearance of ω3 desaturase genes could be a need for increasing tolerance of cells to low temperatures, which may also determine the choice of natural habitat for different microorganisms.

Structural determinants for substrate specificity of ω3 desaturases

ω3 desaturases from different species have different substrate preferences. The ω3 desaturase from *S. kluyveri* recognized the ω3 position of 18-carbon and 20-carbon fatty acids, but the protein desaturated higher amounts of 18-carbon than of 20-carbon fatty acids (Oura and Kajiwara 2008). However, the ω3 desaturase from *P. pastoris* showed broad ω6 fatty acid substrate specificity by its ability to convert all the 18-carbon and 20-carbon ω6 substrates examined to the corresponding ω3 fatty acids, with a high conversion rate that was approximately equivalent (Zhang et al. 2008). As we discussed earlier, the ω3 gene from an EPA-rich fungus, *S. diclina*, encodes a novel ω3 desaturase. The corresponding recombinant protein in a yeast expression system could only desaturate 20-carbon ω6 fatty acid substrates, with a distinct preference for ARA (Pereira et al. 2004). *C. elegans* ω3 desaturase prefers 18-carbon ω6 PUFAs to 20-carbon ω6 PUFAs as substrates (Meesapyodsuk et al. 2000). The substrate specificity of the *M. alpina* 1S-4 ω3 desaturase differs from those of the known fungal ω3 desaturases. It is rather similar to that of *C. elegans* ω3 desaturase, but the *M. alpina* ω3 desaturase can more effectively convert ARA

into EPA when expressed in yeast (Sakuradani et al. 2005). The bifunctional desaturases shows a broad catalytic promiscuity with respect to ω3 desaturation on both C18- and C20-carbon ω6 substrates. *F. moniliforme* ω3 desaturase prefers C18 ω6 over C20 ω6 fatty acids, which is similar to *M. alpina* and *C. elegans* (Damude et al. 2006).

In general, the substrate specificity of ω3 desaturases is determined by the protein structure. The crystal structure of some soluble desaturases, such as the Δ9 stearoyl-acyl carrier protein desaturase from castor seed, the ivy Δ4-16:0-ACP desaturase and a putative acyl-ACP desaturase from *Mycobacterium tuberculosis* H37Rv, have been determined (Dyer et al. 2005; Guy et al. 2007; Lindqvist et al. 1996). The diiron active site of these enzymes is buried within a four-helix core bundle and is positioned alongside a deep, bent, narrow hydrophobic cavity in which the substrate is bound during catalysis. However, due to technical difficulties involved in obtaining large quantities of purified membrane-bound proteins, there is no available information on the three-dimensional structure of membrane-bound desaturases. Moreover, the membrane desaturase active site is suggested to have a fundamentally different architecture from that of the soluble desaturases, perhaps having a cleft into which substrates enter laterally rather than a deep binding cavity into which substrates enter in extended conformation as for the soluble desaturases (Shanklin et al. 2009). Nevertheless, a model previously proposed for the membrane desaturases can shed some light on the structure–function relationship of these enzymes.

A topology model for membrane-bound desaturases was firstly proposed by Joseph E. Stukey in 1990. As shown in Fig. 3, it consists of four membrane-spanning domains with the N- and C-termini as well as the catalytic site in the cytosolic side of the membrane (Stukey et al. 1990). It is easy to understand why the catalytic site should be localized on the cytoplasmic surface of the endoplasmic reticulum, where it has access to substrates and cofactors such as NADH, NADPH, AMP, and ATP. To date, experimental evidence on the topology of membrane desaturases or enzymes that contain the conserved His-motifs can be derived from studies performed in prokaryotes and animals, namely, the acyl-lipid Δ5 desaturase from *Bacillus subtilis* (Diaz et al. 2002), the membrane-bound alkane hydroxylase from *Pseudomonas oleovorans* (van Beilen et al. 1992) and the stearoyl-CoA desaturase 1 from the mouse (Man et al. 2006).

Biochemical topology analysis of membrane-bound fatty acid desaturases from *Aspergillus nidulans* indicated that sufficient determinants of fatty acid desaturase substrate specificity could be narrowed down to a membrane-peripheral region close to the catalytic site defined by conserved histidine-rich motifs in the topology model (Hoffmann et al. 2007). Targeted mutagenesis of amino acid residues near the second histidine box of the *Mucor rouxii* Δ6 desaturase revealed that some of these residues are involved in substrate binding, thus playing a role in substrate preference (Na-Ranong et al. 2006). Several other mutation studies conducted in *Spirulina platensis* Δ6 desaturase (Hongsthong et al. 2004), *Acheta domesticus* Δ9/Δ12 desaturase (Vanhercke et al. 2011) and FAD2-like acetylenases and desaturases (Gagne et al. 2009) also indicate that residues around the first two conserved histidine boxes and between them are important structural determinants for substrate recognition.

For soluble desaturases, the existence of bi- or multi-functional enzymes enables the host organism to obtain various biosynthetic outcomes when the enzymes are expressed in distinct tissues with different available substrates (Dyer et al. 2002). This indicates that the presentation of different substrates influences substrate specificities. Residues presumed to bind the diiron site in both soluble and membrane-bound desaturases are found in the same relative positions in the sequences of enzymes that exhibit diverse specificities and functional outcomes (Shanklin et al. 2009). This implies that the iron-bound oxidant occurs

in fixed positions; thus, substrate factors may also influence the regioselectivity of membrane desaturases. The regioselectivity of a bifunctional 16:0 Δ7/Δ9 desaturase is controlled by its subcellular targeting to different organelles in which the same fatty acid is esterified to different head groups (Heilmann et al. 2004). Thus, the multiple functions of ω3 desaturases from different species may be explained to some extent by enzymatic plasticity when expressed in yeast.

Despite these studies, our understanding of the individual functional domains of ω3 desaturases remains very limited.

Biotechnological use

Although our understanding of ω3 desaturases is still fragmentary, some attempts have been made to manipulate this gene in oleaginous fungi and plants. Oleaginous yeasts can accumulate up to 70% of their dry cell weight as oil (Ratledge and Wynn 2002) but have not been reported as a production platform for VLC-PUFAs. The expression of *F. moniliforme* ω3 desaturase enabled the production of α-linolenic acid (ALA;18:3 ω3) to more than 28 % of total fatty acids in wild-type *Y. lipolytica*, which is the highest level reported in a microbe (Damude et al. 2006). It is higher than previously reported for the expression of nonfungal ω3 desaturases in *S. cerevisiae*, where ALA reached 4.1 % of total fatty acids with *C. elegans* ω3 desaturase (Meesapyodsuk et al. 2000) and 7.8 % of total fatty acids with flax ω3 desaturase (Vrinten et al. 2005). This may indicate that fungal ω3 desaturases have a higher activity than nonfungal ω3 desaturases, and opens the way for producing ω3 PUFAs in *Y. lipolytica* and other oleaginous yeasts.

An oleaginous fungus, *M. alpina* 1S-4, was isolated as a suitable source of ARA; it can produce EPA through the ω3 PUFA biosynthetic pathway when cultured below 20 °C as well as ARA through the ω6 PUFA biosynthetic pathway (Shimiziu et al. 1988; Shinmen et al. 1989). ω3 desaturase-defective mutants Y11 and K1 were isolated from *M. alpina* 1S-4 (Jareonkitmongkol et al. 1992; Sakuradani et al. 2004a). Mutant Y11 is expected to be more suitable for ARA production: in a 5-l jar fermentor, the ARA content in Y11 reached 1.48 mg/ml (118 mg/g of dry mycelia), compared with 0.95 mg/ml (86 mg/g of dry mycelia) for wild-type *M. alpina* 1S-4 (Sakuradani et al. 2004b). The mutation sites in the two desaturase-defective mutants were identified on the ω3 desaturase genes (Sakuradani et al. 2009) and the essential regions for the desaturase activity were mapped through identification of the mutation sites. Most importantly, the overexpression of this gene in *M. alpina* 1S-4 resulted in a maximum yield of EPA, reaching 40 % of total fatty acids (Ando et al. 2009). This not only proves the very important role that ω3 desaturase plays in converting ω6 fatty acids into ω3 fatty acids in this organism, but also implies that it could potentially be used as an alternative source of important VLC-PUFAs.

Fungal ω3 desaturases have also been expressed in oleaginous plants. Seed-specific expression of *F. moniliforme* ω3 desaturase in soybean resulted in production of ALA to levels as high as 70.5 % of total fatty acids, considerably higher than 53.2 % of total fatty acids found in linseed oil; thus, this could represent a competitive alternative to linseed oil. Meanwhile, ω3/ω6 ratio was increased dramatic in soybeans, and was as high as 22:1, or 6-fold higher than linseed oil (Damude et al. 2006). Recently, ALA content has been increased significantly in another important staple food. ω3 desaturase genes cloned from rice and soybean were introduced into rice under the control of an endosperm-specific promoter, the ALA contents in the transgenic rice seeds was increased from 0.36 to 8.57 and 10.06 mg/g, respectively, which was 23.8- and 27.9-fold higher than that of non-transformants (Liu et al. 2012).

Fungal ω3 desaturases are also key components in successful attempts to make ω3 VLC-PUFAs in plants. An engineered pathway using seven fatty acid biosynthesis genes resulted in the accumulation of up to 15% DHA in the seed oil of *Arabidopsis thaliana*, a level that exceeds the 12 % level generally found in commodity bulk fish oil (Petrie et al. 2012). A high ω3/ω6 ratio was also observed, and it was likely due to the presence of the broad-specificity *P. pastoris* ω3 desaturase. Very recently, a large systematic evaluation of the role of thirteen different genes in twelve different combinations for their capacity to synthesize ω3 VLC-PUFAs in *A. thaliana* also indicates the critical role for fungal ω3 desaturases in directing the flux of fatty acids towards the desired targets of EPA and DHA (Ruiz-Lopez et al. 2013).

The combination of high activity and broad substrate specificities of ω3 desaturases from fungi makes these enzymes particularly attractive for biotechnological use in oleaginous species.

In addition, there are some interesting genetic studies and applications regarding *C. elegans* ω3 desaturase. Kang have used *C. elegans* fat-1 gene encoding ω3 desaturase to generate a transgenic mouse which are capable of converting ω6 to ω3 fatty acids (Kang et al. 2004). It is being used widely as a new tool for studying the benefits of ω3 fatty acids and the molecular mechanisms of their action. In transgenic mice, fat-1-induced decreases in the ω6/ω3 ratio result in apoptosis in lung cancer cells (Xia et al. 2005), prostate cancer cells (Lu et al. 2008), and epithelial cells of the colon (Jia et al. 2008) and inhibition of neuronal apoptosis (Ge et al. 2002). One the other hand, the generation of the transgenic mouse indicates that this technology might be adapted to enrich these highly valuable fatty acids in animal products such as meat, milk, and eggs.

Conclusion and future perspectives

Over the last few decades, major advances have been made in the cloning and identification of ω3 fatty acid desaturase genes from an array of different microorganisms. Although phylogenetic analysis of desaturases from different species, together with the membrane topology prediction, has helped us gain some insights into the structure–function relationship, our understanding of this membrane desaturase is still limited. What remains to be carried out is the structural characterization of the purified membrane-bound desaturases. To achieve this, two major issues need to be resolved. First, a system for expressing a functional desaturase needs to be established; and second, a method of purification of the active protein with yields sufficient for structural analysis needs to be developed.

Our group has successfully expressed high amounts of recombinant integral membrane desaturases from *M. alpina* ATCC#32222 in the eukaryotic expression host *P. pastoris* and determined Fos-Choline 16 to be a suitable detergent for extraction; this represents a critical step towards the structural elucidation of membrane fatty acid desaturases (Chen et al. 2013; Wang et al. 2011).

On the other hand, there is little information available on how the expression of this desaturase is regulated. There is evidence that unsaturated fatty acid homeostasis in many organisms is achieved by feedback regulation of fatty acid desaturase gene transcription through signaling pathways that are controlled by sensors embedded in cellular membranes (Aguilar and de Mendoza 2006). The regulation of ω3 desaturases is likely to adopt a similar strategy, but many studies remains to be conducted in this field.

As ω3 desaturases play important roles in the biosynthesis of very-long-chain polyunsaturated fatty acids, a better understanding of their structure–function relationship not only opens up further possibilities to manipulate the activity and substrate specificity of

fatty acid enzymes, but could also lead to the commercial production of novel and highly valuable, industrially important fatty acids.

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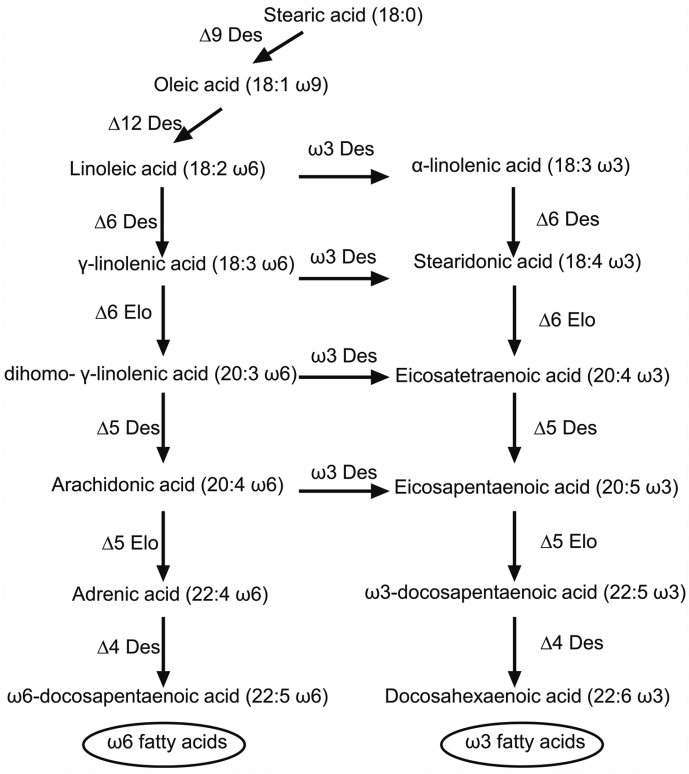

Fig. 1.

The major pathway of fatty acids biosynthesis in eukaryotic microorganisms. *ΔN Des* delta *N* fatty acid desaturase, *ΔN Elo* delta *N* fatty acid elongase, *ω3 Des* ω3 fatty acid desaturase

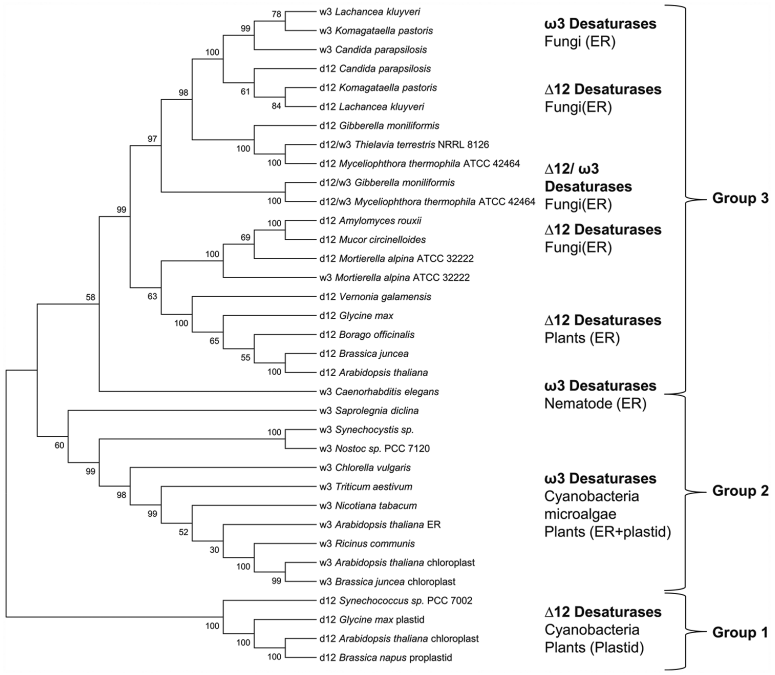

Fig. 2.

Neighbor-joining tree showing the relationship among different ω3 desaturases and Δ12 desaturases. The Poisson model was used as the substitution model and gaps were treated using the method of pairwise deletion

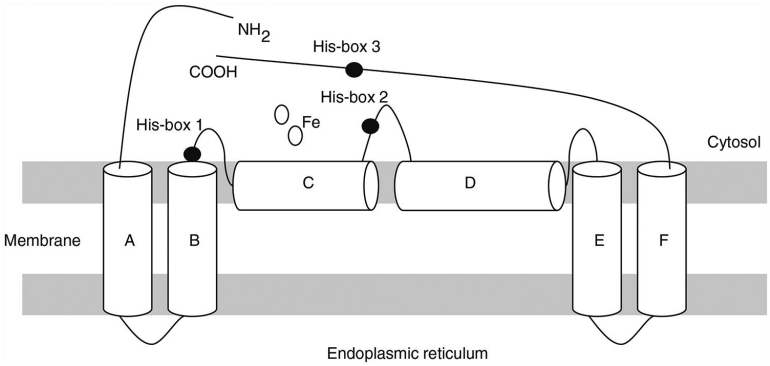

Fig. 3.

Topology model of ω3 desaturase from *Mortierella alpina* ATCC#32222. *Cylinders A–F* indicate membrane-spanning helices, and the black circles indicate three conserved histidine-rich motifs. Topology prediction was based on HMMTOP, TMPRED, and TMHMM algorithms

Table 1

Conserved histidine-box motifs in ω3 desaturases from different organisms

	Organism	H-Box 1	H-Box 2	H-Box 3
Fungi	*Mortierella alpina*	HECGH	HSKHH	HQCHH
	Lachancea kluyveri	HECGH	HSKHH	HVLHH
	Komagataella pastoris	HECGH	HAKHH	HVLHH
	Candida parapsilosis	HECGH	HAKHH	HVLHH
	Saprolegnia diclina	HDCGH	HRHHH	HQVHH
Plants	*Arabidopsis thaliana* [a]	HDCGH	HRTHH	HVIHH
	Arabidopsis thaliana [b]	HDCGH	HRTHH	HVIHH
	Triticum aestivum	HDCGH	HRTHH	HVIHH
	Brassica juncea [b]	HDCGH	HRTHH	HVIHH
	Ricinus communis	HDCGH	HRTHH	HVIHH
	Nicotiana tabacum	HDCGH	HKTHH	HVIHH
Cyanobacteria	*Synechocystis sp.*	HDCGH	HRTHH	HVAHH
	Nostoc sp. PCC 7120	HDCGH	HRTHH	HVAHH
Microalgae	*Chlorella vulgaris*	HDCGH	HRTHH	HVVHH
Nematode	*Caenorhabditis elegans*	HDCLH	HKLHH	HVAHH

[a] Microsomal

[b] Plastidial