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Substrate Specificity and Regioselectivity of Δ12 and ω3 Fatty Acid Desaturases from *Saccharomyces kluyveri*

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Δ12 and ω3 fatty acid desaturases are key enzymes in the synthesis of polyunsaturated fatty acids (PUFAs), which are important constituents of membrane glycerolipids and also precursors to signaling molecules in many organisms. In this study, we determined the substrate specificity and regioselectivity of the Δ12 and ω3 fatty acid desaturases from *Saccharomyces kluyveri* (*Sk*-FAD2 and *Sk*-FAD3). Based on heterologous expression in *Saccharomyces cerevisiae*, it was found that *Sk*-FAD2 converted C16–20 monounsaturated fatty acids to diunsaturated fatty acids by the introduction of a second double bond at the $\nu+3$ position, while *Sk*-FAD3 recognized the ω3 position of C18 and C20. Furthermore, fatty acid analysis of major phospholipids suggested that *Sk*-FAD2 and *Sk*-FAD3 have no strong substrate specificity toward the lipid polar head group or the *sn*-positions of fatty acyl groups in phospholipids.

Key words: fatty acid desaturase; *Saccharomyces kluyveri*; polyunsaturated fatty acid

Δ12 and ω3 fatty acid desaturases introduce the second and third double bonds respectively in the biosynthesis of PUFAs such as linoleic acid (C18:2) and α-linolenic acid (α-C18:3). These fatty acids are crucial constituents of membrane glycerolipids and precursors to signaling molecules in higher organisms such as animals and plants.[1,2] Mammals lack these enzymes and thus must obtain 18-carbon polyunsaturated fatty acids (C18-PUFAs) from the diet.[1] Desaturation and elongation of these fatty acids produce C20–C22 PUFAs, which become membrane components and precursors of eicosanoids.[2] In contrast, a wide range of organisms other than mammals are capable of producing C18:2 and α-C18:3, and genes encoding Δ12 and ω3 fatty acid desaturases have been cloned from many organisms.[3,4] However, information about the substrate specificities and regioselectivities of these enzymes is limited.

Membrane-bound fatty acid desaturases of eukaryotes are non-heme iron containing oxygen-dependent enzymes involved in regioselective introduction of double bonds in fatty acyl aliphatic chains. Three regioselective classes have been observed for these fatty acid desaturases. Δx fatty acid desaturases introduce a double bond between the x and x + 1 carbons from the carboxyl end of fatty acids. ωx fatty acid desaturases introduce a double bond between the x and x + 1 carbons from the methyl terminus of fatty acids. And $\nu+$x fatty acid desaturases use a preexisting double bond as a reference point and introduce a double bond between the x and x + 1 carbons from the olefinic carbon of fatty acids.[5]

Previously, Δ12 fatty acid desaturases were thought to have the ability to convert monounsaturated fatty acids to diunsaturated fatty acids by the introduction of a second double bond at the Δ12 position; these enzymes were classified as Δx fatty acid desaturases.[3] However, researchers recently reported that Δ12 fatty acid desaturases from the peanut *Arachis hypogaea L.* and the nematode *Caenorhabditis elegans* desaturated nonadecenoic acid [C19:1 (Δ10)] to nonadecadienoic acid [C19:2 (Δ10, 13)], suggesting that these enzymes have regioselectivity that yields a double bond three carbon atoms distal to an existing double bond relative to the carboxyl terminus.[5,6] Furthermore, the regioselectivity of ω3 fatty acid desaturases from *Brassica napus* and *C. elegans* has also been characterized.[7,8] Both of these ω3 fatty acid desaturases can introduce a double bond not only at the ω3 position of C18, but also of C20. Based on these results, it was confirmed that these enzymes are ω3 fatty acid desaturases.

With regard to the acyl substrates of Δ12 and ω3 fatty acid desaturases, on the other hand, these enzymes are known to act on lipid-linked substrates.[9–12] Biochemical studies of fatty acid desaturases from plants and fungi strongly suggest that these Δ12 fatty acid desaturases act on both positions (*sn*-1 and *sn*-2) of phosphatidylcholine (PC).[13–15] More recently, Domergue *et al.* (2003) found that several plant Δ12 fatty acid desaturases are able to introduce a double bond at the *sn*-positions of acyl groups in most glycerolipids and phospholipids.[16]

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Previously, we succeeded in the first clonings of yeast Δ12 and ω3 fatty acid desaturase genes from the PUFA-producing yeast *Saccharomyces kluyveri* (*Sk-FAD2* and *Sk-FAD3* respectively).[17,18] Disruption of these genes indicated that synthesis of PUFAs in *S. kluyveri* is conducted only by *Sk*-FAD2 and *Sk*-FAD3. In this report, we describe efforts to elucidate the substrate specificity and regioselectivity of the Δ12 and ω3 fatty acid desaturases from *S. kluyveri*. Based on expression of the *Sk-FAD2* and *Sk-FAD3* genes in *S. cerevisiae*, it was found that the *Sk*-FAD2 protein is capable of converting C19:1 (Δ10) to C19:2 (Δ10, 13), and that the *Sk*-FAD3 protein is able to recognize the ω3 position of C18 and C20. Fatty acid analysis of major phospholipids (phosphatidylcholine, PC, phosphatidylethanolamine, PE, phosphatidylserine, PS, and phosphatidylinositol, PI) in *S. kluyveri* suggested that *Sk*-FAD2 and *Sk*-FAD3 proteins act on the fatty acyl groups linked to all of these phospholipids, although the fatty acyl group of PS is poorly recognized as a substrate. Positional analysis of fatty acids in PC and PE from *S. kluyveri* revealed that the *Sk*-FAD2 and *Sk*-FAD3 proteins can act on the *sn*-1 and *sn*-2 positions in these phospholipids, although *Sk*-FAD3 does not recognize the fatty acyl group at the *sn*-2 position in PE efficiently.

Materials and Methods

Strains, media, plasmids, and fatty acids. *S. kluyveri* IFO1893 cells were grown in complete minimal (CM) medium for total lipid extraction. *S. cerevisiae* IFO10150 [*MAT*a, *ste-VC9*, *ura3-52 trp1-289 his3-Δ1 leu2-3,112*] was used as a recipient for transformation.[19] Plasmids pYSk-FAD2 and pYSk-FAD3, used for *Sk-FAD2* and *Sk-FAD3* expression respectively, were constructed as described previously.[17,18] For induction of gene expression in *S. cerevisiae*, yeast cells were cultivated in CM (raffinose) medium without uracil, to which was added 1/100 vol of 20% galactose solution, various fatty acids to a final concentration of 200 mg/l, and 0.1% IGEPAL CA-630 (Sigma-Aldrich Japan, Tokyo, Japan).[20] Fatty acids and fatty acid methyl esters were purchased from Nu-Chek-Prep (Elysian, MN, USA) and Sigma-Aldrich Japan. All fatty acids used were of known purity (typically >99%).

Total lipid and fatty acid analysis. Cultured yeast cells were harvested and total lipids were extracted with chloroform/methanol (1:2, v/v). The lipids were then methylated with 1% sulfuric acid in methanol at 90 °C for 2 h, as described previously.[21] The resulting fatty acid methyl esters (FAMEs) were extracted with hexane. The FAMEs were analyzed by gas-liquid chromatography (GLC: GC-18A, Shimadzu, Kyoto, Japan) with a 0.25 mm × 25 m HR-SS-10 capillary column (Shinwa Chemical Industries, Kyoto, Japan). The initial column temperature of 150 °C was held for 2 min and raised 2 °C/min to 180 °C and then held for 2 min. A FAME mixture used as a lipid standard was purchased from Sigma (St. Louis, MO, USA). Fatty acid 4,4-dimethyloxazoline (DMOX) derivatives were prepared from the FAMEs by evaporating the hexane phase, resuspending the residue in 0.5 ml of 2-amino-2-methylpropanol, and heating overnight at 180 °C.[22] After cooling, the DMOX derivatives were dissolved in 4 ml of dichloromethane and washed twice with 1.5 ml of distilled water. The dichloromethane solution was evaporated under a stream of nitrogen, and the residue was dissolved in hexane for injection. Analyses of DMOX derivatives were conducted using the same equipment as for GLC analysis and gas chromatography-mass spectrometry (GC-MS: QP5000, Shimadzu, Kyoto, Japan) operating at an ionization voltage of 70 eV, with a scan range of 40–400 kDa. The initial column temperature of 150 °C was held for 2 min and raised 10 °C/min to 190 °C and then raised 2 °C/min to 210 °C and held for 2 min.

Phospholipid analysis. In phospholipid analysis, total lipids were extracted from log-phase cells (OD_{600} = 1.0–1.5) with chloroform/methanol (1:2, v/v). Individual phospholipids, PC, PE, PS, and PI were separated by two-dimensional thin layer chromatography (TLC) using chroloform/methanol/7M ammonia (65:30:4, v/v) for the first and chloroform/methanol/acetic acid/water (170:25:25:4, v/v) for the second direction. The different spots were scraped off, and lipids were extracted with chloroform/methanol/water (1:2:0.8, v/v) and then methylated using 1% sulfuric acid in methanol at 90 °C for 2 h. The resulting FAMEs were analyzed by GLC, as described previously. The positional distribution of fatty acids was determined by phospholipase A_2 cleavage of PC and PE. The phospholipids were resuspended in a solution containing 100 mM Tris–HCl, pH 8.0, 10 mM $CaCl_2$, 0.05% Triton X-100, and 15 units of phospholipase A_2 from *Naja mossambica mossambica* (Sigma-Ardrich Japan, Tokyo, Japan) and incubated at 37 °C for 4 h. Lysophospholipids were then separated from free fatty acids by TLC with chloroform/methanol/water (65:25:4, v/v). The different spots were scraped off, and lipids were extracted with chloroform/methanol/water (1:2:0.8, v/v) and then methylated using 1% sulfuric acid in methanol at 90 °C for 2 h. The resulting FAMEs were analyzed by GLC as described previously.

Results and Discussion

Characterization of substrate specificity and regioselectivity of Δ12 fatty acid desaturase from S. kluyveri

In order to characterize further the substrate specificity and regioselectivity of *Sk*-FAD2, the IFO10150 strain harboring pYSk-FAD2 was grown in CM (raffinose) medium supplemented with 0.2% galactose and 200 mg/l of each fatty acid substrate. This transformant was grown at 30 °C for four generations, and then total lipids were extracted and subjected to GLC analysis. Total

Table 1. Conversion Rates of Fatty Acids by *S. cerevisiae* IFO10150 Expressing *Sk-FAD2*

Substrate	Substrate (%)*	Product	Product (%)*	Conversion rate (%)*
C16:1 (Δ9)	30.5 ± 0.4	C16:2 (Δ9, 12)	4.8 ± 0.1	13.6 ± 0.3
C18:1 (Δ9)	18.9 ± 0.3	C18:2 (Δ9, 12)	12.5 ± 0.2	39.8 ± 0.4
C19:1 (Δ10)	13.7 ± 0.2	C19:2 (Δ10, 13)	2.2 ± 0.0	13.6 ± 0.0
C20:1 (Δ11)	12.5 ± 0.9	C20:2 (Δ11, 14)	0.3 ± 0.0	0.9 ± 0.3

*Results were determined from the peak areas of methyl esters. Values are means of three independent analyses.

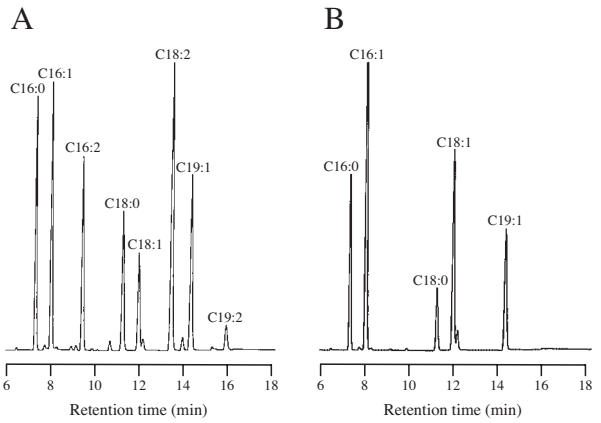

Fig. 1. GLC Analysis of Fatty Acid Patterns in Total Lipids from *S. cerevisiae* Transformants.

The IFO10150 strain harboring pYSk-FAD2 (A) and pYES2 (B) was cultured in CM (raffinose) medium supplemented with 0.2% galactose and 200 mg/l nonadecenoic acid at 30 °C. C16:0, palmitic acid; C16:1, palmitoleic acid; C16:2, hexadecadienoic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C19:1, nonadecenoic acid; C19:2, nonadecadienoic acid.

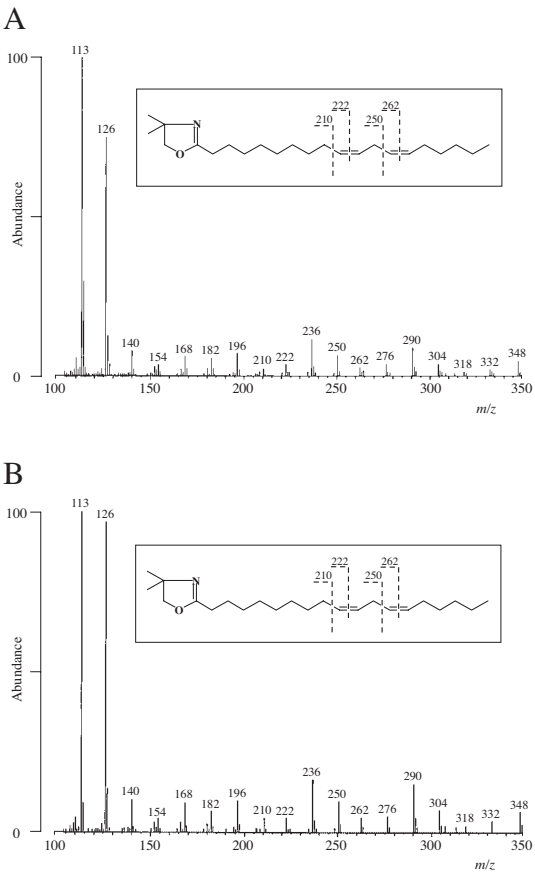

Fig. 2. GC–MS Analysis of the DMOX Derivative Fatty Acid.

A, The spectrum of the DMOX derivative of the new fatty acid shown in Fig. 1 as C19:2 indicated that it was a nonadecadienoic acid [C19:2 (Δ10, 13)] derivative. B, The spectrum of the DMOX derivative prepared from C19:2 (Δ10, 13) (Nu-Chek-Prep, Elysian, MN, USA). The details of the interpretation of the spectrum are given in the text. The deduced structure of the fatty acid derivative is shown above the spectrum.

lipids from IFO10150 harboring pYES2 was also subjected to GLC analysis as a control. The fatty acid substrates tested and the products of desaturation in the *S. cerevisiae* strain carrying pYSk-FAD2 are listed in Table 1. All of the fatty acid substrates used in this experiment were converted to $\nu + 3$ desaturation products. The positions of newly formed double bonds in the products of desaturation derived from the tested fatty acid substrates were determined by GC–MS analysis of DMOX derivatives. An example of this analysis is shown in Fig. 1 and Fig. 2. In the case of cultures supplied with C19:1 (Δ10), one major peak, as well as C16:2 (Δ9, 12) and C18:2 (Δ9, 12) peaks was observed in the transformant carrying pYSk-FAD2 (Fig. 1). The mass spectrum of the fatty acid DMOX derivative exhibited a peak at $m/z = 348$ expected for its molecular ion and peaks at $m/z = 210$, 222, 250, and 262, consistent with double bonds at the Δ10 and Δ13 positions of the fatty acid (Fig. 2A). This mass spectrum pattern was consistent with that of the DMOX derivative prepared from C19:2 (Δ10, 13) purchased from Nu-Chek-Prep (Elysian, MN, USA) (Fig. 2B). Based on these analyses, this new peak was identified as C19:2 (Δ10, 13), not C19:2 (Δ10, 12), the Δ12 desaturation product of C19:1 (Δ10). These results suggest that *Sk*-FAD2 catalyzes the introduction of a double bond at the $\nu + 3$ position of enoic fatty acids and not at the Δ12 position. They also indicate that *Sk*-FAD2 converted the monounsaturated fatty acids examined to diunsaturated fatty acids by the introduction of a second double bond at the $\nu + 3$ position, and that C18:1 (Δ9) was the most favorable substrate among those tested. The *Sk*-FAD2 protein introduced a double bond at the $\nu + 3$ position of monounsaturated fatty acids, C16:1 (Δ9), C18:1 (Δ9), C19:1 (Δ10), and C20:1 (Δ11). These results agree with those for Δ12 fatty acid desaturases from the peanut *A. hypogaea L.* and the nematode *C. elegans*.[5,6] Therefore, so-called Δ12 fatty acid desaturases from other eukaryotic organisms can be also classified not as Δ12 fatty acid desaturases, but as $\nu + 3$ fatty acid desaturases.

Characterization of the substrate specificity and regioselectivity of the ω3 fatty acid desaturase from S. kluyveri

In order to characterize the substrate specificity and

Table 2. Conversion Rates of Fatty Acids by *S. cerevisiae* IFO10150 Expressing *Sk-FAD3*

Substrate	Substrate (%)*	Product	Product (%)*	Conversion rate (%)*
C18:1 (Δ9)	68.8 ± 3.6	C18:2 (Δ9, 15)	0.4 ± 0.0	0.7 ± 0.1
C18:2 (Δ9, 12)	50.5 ± 0.9	C18:3 (Δ9, 12, 15)	14.2 ± 0.5	22.0 ± 0.9
C18:3 (Δ6, 9, 12)	35.1 ± 0.4	C18:4 (Δ6, 9, 12, 15)	3.9 ± 0.4	10.0 ± 0.8
C20:1 (Δ11)	9.6 ± 0.5	C20:2 (Δ11, 17)	0.1 ± 0.0	0.9 ± 0.3
C20:2 (Δ11, 14)	17.5 ± 3.5	C20:3 (Δ11, 14, 17)	1.1 ± 0.6	5.9 ± 2.2
C20:3 (Δ8, 11, 14)	22.9 ± 0.2	C20:4 (Δ8, 11, 14, 17)	0.9 ± 0.2	3.9 ± 0.8
C20:4 (Δ5, 8, 11, 14)	34.2 ± 3.1	C20:5 (Δ5, 8, 11, 14, 17)	0.6 ± 0.0	1.6 ± 0.1

*Results were determined from the peak areas of methyl esters. Values are means of three independent analyses.

regioselectivity of *Sk*-FAD3, the IFO10150 strain harboring pYSk-FAD3 was grown under conditions identical to those for pYSk-FAD2 described above. Total lipids were extracted and subjected to GLC analysis. The fatty acid substrates tested and the products of desaturation in *S. cerevisiae* strain carrying pYSk-FAD3 are listed in Table 2. All of the fatty acid substrates used in this experiment were converted to ω3 desaturation products. This result indicated that *Sk*-FAD3 catalyzes the introduction of a double bond at the ω3 position not only of C18:2 (Δ9, 12), but also of C18:1 (Δ9) and γ-C18:3 (Δ6, 9, 12). Additionally, *Sk*-FAD3 was found to recognize the ω3 position of several 20-carbon UFAs as a substrate. Previously, the substrate specificity and regioselectivity of the ω3 fatty acid desaturases from *C. elegans* (FAT1) and *B. napus* (*Bn*-FAD3) were characterized by Meesappyodsk *et al.* (2000) in detail.[7] Like FAT1 and *Bn*-FAD3, the *Sk*-FAD3 protein desaturated higher amounts of C18 than of C20. Additionally, *Sk*-FAD3 has a preference for dienoic fatty acids such as C18:2 (Δ9, 12) and C20:2 (Δ11, 14) as substrates. The substrate preference of *Sk*-FAD3 is similar to that of *Bn*-FAD3, but different from that of FAT1 which prefers trienoic fatty acids such as γ-C18:3 (Δ6, 9, 12) and C20:3 (Δ8, 11, 14). Based on these results, the *Sk*-FAD3 protein appears to share greater functional similarity with plant desaturases than with those of animals.

Characterization of the acyl substrate specificity of Δ12 and ω3 fatty acid desaturases from S. kluyveri

In order to determine the acyl substrate specificity of Δ12 and ω3 fatty acid desaturases from *S. kluyveri*, the fatty acid distribution in different phospholipids in the wild-type strain of *S. kluyveri* was analyzed. After extraction and separation of the major phospholipid classes, the fatty acid patterns of PC, PE, PS, and PI were analyzed (Fig. 3). Desaturase activities were calculated as (C18:2 + α-C18:3)/(C18:1 + C18:2 + α-C18:3) × 100 for *Sk*-FAD2, and as α-C18:3/(C18:2 + α-C18:3) × 100 for *Sk*-FAD3 to use as values corresponding to the percentage of total fatty acids (Fig. 3). As clearly shown in Fig. 3, these fatty acid desaturases were not specific for any one phospholipid, similar to plant Δ12 fatty acid desaturases. However, they did appear to have some substrate preference. For

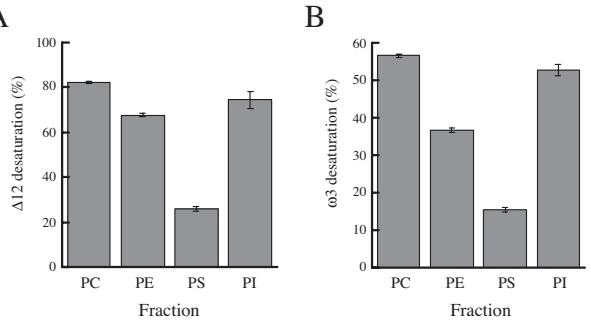

Fig. 3. Δ12 (A) and ω3 (B) Desaturation in Different Phospholipids in the *S. kluyveri* IFO1893 Strain.

Desaturase activities were calculated as (C18:2 + α-C18:3)/(C18:1 + C18:2 + α-C18:3) × 100 for *Sk*-FAD2, and as α-C18:3/(C18:2 + α-C18:3) × 100 for *Sk*-FAD3, using values corresponding to the percentage of total fatty acids.

PC, PE, and PI, C18:2 and α-C18:3 represented about 35% of total fatty acids, while for PS, these PUFAs represented only 11.8% of total fatty acids. Additionally, Δ12 and ω3 desaturation was rather low for PS (25.9% and 15.5% respectively) (Fig. 3). These results suggest that these fatty acid desaturases do not recognize the fatty acyl group in PS efficiently.

In addition, the fatty acid profiles of the *sn*-1 and *sn*-2 positions of PC and PE were determined (Fig. 4). C18:2 and α-C18:3 were accumulated at both *sn*-positions of PC, suggesting that these enzymes are capable of acting on both positions of this phospholipid. On the other hand, for PE, Δ12 and ω3 desaturation at the *sn*-2 position was lower than at the *sn*-1 position; in particular, ω3 desaturation at the *sn*-2 position was rather low (19.4%) (Fig. 4). These results suggest that *Sk*-FAD2 and *Sk*-FAD3 are capable of acting on both positions (*sn*-1 and *sn*-2) of PC and PE, but that in the case of PE, these enzymes prefer the *sn*-1 position. It is known that two more desaturation steps (Δ5 and Δ6) catalyzed by the Δ5 and Δ6 fatty acid desaturases from lower plants, fungi, worms, and algae occur predominantly at the *sn*-2 positions of PC.[16] However, as our results show, the Δ12 and ω3 fatty acid desaturases from *S. kluyveri* appear to have no strong specificity toward the lipid polar head group or the *sn*-positions, similar to plant Δ12 and ω3 fatty acid desaturases. Therefore, at least the Δ12 and ω3 fatty acid desaturases of a member

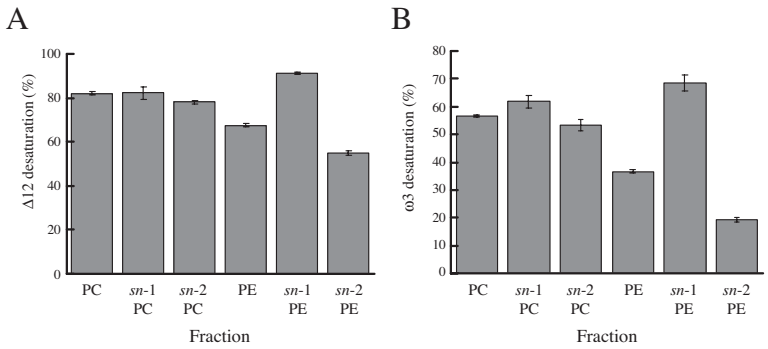

Fig. 4. Δ12 (A) and ω3 (B) Desaturation at the *sn*-1 and *sn*-2 Positions in PC and PE in the *S. kluyveri* IFO1893 Strain.
Desaturase activities were calculated as (C18:2 + α-C18:3)/(C18:1 + C18:2 + α-C18:3) × 100 for *Sk*-FAD2, and as α-C18:3/(C18:2 + α-C18:3) × 100 for *Sk*-FAD3, using values corresponding to the percentage of total fatty acids.

of the fungi, *S. kluyveri*, can recognize lipid substrates in a manner different from Δ5 and Δ6 fatty acid desaturases.

Acknowledgments

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