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Detection of Acyl-CoA Derivatized with Butylamide for *in vitro* Fatty Acid Desaturase Assay

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Abstract: Membrane-bound fatty acid desaturases acting on acyl-CoA contribute to the biosynthesis of unsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid in higher organisms. We propose a simplified method for measuring the desaturase activity that combines the *in vitro* reaction by desaturase-expressing yeast cell homogenate and the detection of acyl-CoA product as butylamide derivatives by gas chromatography. To set up the *in vitro* reaction, we traced the *in vivo* activity of rat liver Δ6 fatty acid desaturase (D6d) expressed in the yeast, *Saccharomyces cerevisiae*, and determined the time taken for the D6d activity to reach its maximum level. The cell homogenate of yeast expressing the maximum D6d activity was made to react *in vitro* with linoleoyl-CoA to generate the D6d product, γ-linolenoyl-CoA. This product was successfully detected as a peak corresponding to γ-linolenoyl butylamide on gas chromatography. This procedure, with low background expression, using non-labeled acyl-CoA as substrate, will contribute toward developing a simple *in vitro* desaturase assay. It will also help in elucidating the functions of membrane-bound fatty acid desaturases with various substrate specificities and regioselectivities.

Key words: fatty acid desaturase, heterologous expression, butylamide derivative, *in vitro* reaction

1 Introduction

Unsaturated fatty acids such as γ-linolenic acid (GLA), arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid form the hydrophobic component of biological membranes and play a role in regulating the membrane fluidity. Some of the polyunsaturated fatty acids are also precursors of eicosanoids, including prostaglandins and thromboxanes that play important roles in the maintenance of homeostasis in higher organisms[1].

Unsaturated fatty acids are generated through desaturation and elongation of the carbon chain backbone of fatty acid substrates[2]. Various types of fatty acid desaturases with different substrate specificities and regioselectivities are found both in prokaryotes and eukaryotes[3–5]. The molecular structure and enzyme characteristics of water-soluble desaturases from cyanobacteria and higher plants, acting on fatty acids bound to acyl carrier protein, have been thoroughly elucidated[6]. Besides, the enzymatic activity of membrane-bound desaturases from fungi, algae, plants, and animals that recognize fatty acids bound to coenzyme A (CoA), or associated with glycerides, is determined *in vitro*, most commonly by using microsome fractions prepared from cells or tissues, and radiolabeled substrate[7, 8]. However, these assays often involve intricate experimental setup.

Cloned membrane-bound desaturases have been characterized *in vivo* by gene disruption[9, 10], and/or heterologous expression analysis in bacteria, fungi, and higher plants[11–13]. The budding yeast, *Saccharomyces cerevisiae*, predominantly contains saturated and mono-unsaturated fatty acids with 16 and 18 carbon atoms[14]. This is advantageous since exogenous fatty acid substrates and products can be clearly discriminated from the endogenous fatty acids[15, 16]. For instance, Δ6 fatty acid desaturase (D6d) from rat liver has been expressed, identified, and characterized using the yeast expression system, where linoleic acid (LA, 18:2 Δ9, 12) and α-linolenic acid (18:3 Δ9, 12, 15) were converted into GLA (18:3 Δ6, 9, 12) and stearidonic acid (18:4 Δ6, 9, 12, 15) respectively[17], none of which were present endogenously. However, *in vivo* assays cannot precisely determine the chemical kinetics as the amount of enzyme expressed and the amount of substrate incorporated cannot be measured accurately.

In this study, an *in vitro* desaturase reaction was carried

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out using cell homogenate from yeast overexpressing D6d and unlabeled acyl-CoA. After specific butylamidation of the acyl-CoA product, acyl butylamide was detected by gas chromatography. This method could serve as a non-radioactive assay for fatty acid desaturase from different sources.

2 Experimental procedures

2.1 Microorganisms, culture media, and reagents

Transformants of *Escherichia coli* DH5α were selected and cultivated in LB medium (0.5% yeast extract, 1% NaCl, 1% Bacto tryptone, 2% agar for plate) containing 50 μg/mL ampicillin at 37℃ with rotary shaking at 120 rpm. Transformants of *S. cerevisiae*, INVSc1 (Invitrogen, Carlsbad, CA, USA) were selected on SD without Ura agar medium (0.67% yeast nitrogen base, 0.19% yeast synthetic dropout medium without uracil, 2% D-glucose, 2% agar) and cultivated in SCT without Ura medium (0.67% yeast nitrogen base, 0.19% yeast synthetic dropout medium without uracil, 4% raffinose, 0.1% tergitol) or YPD medium (2% polypeptone, 1% yeast extract, 2% D-glucose) at 28℃, with rotary shaking at 160 rpm. Fatty acids were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Cayman Chemical (Ann Arbor, MI, USA). Other guaranteed reagents were obtained from Nacalai Tesque (Kyoto, Japan), Sigma-Aldrich, Toyobo (Osaka, Japan), or Wako Chemicals (Osaka, Japan), unless otherwise indicated.

2.2 Expression of rat D6d gene in yeast

A FLAG DNA fragment was synthesized by PCR amplification with the TaKaRa Ex Taq (Takara, Kyoto, Japan) and oligonucleotide primers (5'-GCAAAGCTTAAGATGGA*CTATAAGGATGATGATGAC*-3' and 5'-CGTGGTACCCTT*GTCATCATCATCCTTATAG*-3', where these primers can hybridize with each other at nucleotide regions indicated using italics, and underlined regions are *Hind* III and *Kpn* I recognition sites respectively) using 10 cycles of 95℃ for 30 s, 50℃ for 30 s, and 74℃ for 30 s without template. The fragment was subcloned in the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *E. coli* DH5α (pGEM-FLAG). The rat D6d gene (DDBJ accession number AB021980)[17] was amplified from stock plasmid with the KOD-Dash DNA polymerase (Toyobo) and primers (5'-ACAGGTACC*ATG*GGGAAGGGAGGTAACCAG-3' and 5'-GTCTCTAGAT*TCA*TTTGTGGAGGTAGGCATCC-3', where underlined regions are *Kpn* I and *Xba* I recognition sites, and italicized regions are translation initiation and termination codons respectively), using 30 cycles of 95℃ for 30 s, 68℃ for 2 s and 74℃ for 30 s, and was digested with *Kpn* I and *Xba* I. The product was ligated into *Kpn* I/*Spe* I-digested pGEM-FLAG and the plasmid was transformed into *E. coli* DH5α (pGEM-FLAG-D6d). The FLAG-D6d fragment was obtained by digestion of pGEM-FLAG-D6d with *Hind* III and *Eco*R I and was ligated into the yeast expression vector pYES2 (Invitrogen). The nucleotide sequences of all plasmids were determined using BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, Carlsbad, CA, USA) with T7, SP6, and other appropriate primers on an ABI PRISM 3130x1 genetic analyzer (Life Technologies). The desaturase expression vector was introduced into *S. cerevisiae* INVSc1 by using the lithium acetate method[18]. Transformants were selected on SD without Ura agar plates and cultivated at 28℃ for 12 h with rotary shaking at 160 rpm in 3 mL of SCT without Ura medium. One milliliter of this preculture was transferred to 15 mL of SCT without Ura medium supplemented with linoleic acid (LA) at a concentration of 0.25 mM and cultivated at 28℃ for 6 h. After addition of galactose (2%, w/v) and further cultivation for another 16 h, yeast cells were harvested by centrifugation.

2.3 *In vitro* desaturase reaction

The yeast cells from 1 mL of induced culture were washed with distilled water and suspended at $OD_{600} = 100$ in 50 mM Tris-HCl (pH 7.5) containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride and 4.7 μM pepstatin A. Glass beads (particle size of 0.5 mm) were added to the suspension in the same volume, and the yeast cells were disrupted by eight rounds of vigorous vortex for 30 s and chilling on ice for 30 s. The homogenate was centrifuged at 5,000 × *g* for 10 min and the resultant supernatant was centrifuged further at 100,000 × *g* for 1 h at 4℃ to obtain the microsomal fraction as precipitate. Protein concentrations of the homogenate and the microsomes were measured by using the BCA protein assay reagent (Thermo Scientific Fermentas, Carlsbad, CA, USA)[19]. The reaction mixture for the *in vitro* desaturation was composed of 50 mM potassium phosphate (pH 7.5), 2 mM NADH, 0.1 mM linoleoyl-CoA, and 2 mg-protein/mL homogenate or microsomes. After incubating the mixture at 30℃ for 30 min with reciprocal shaking at 150 rpm, hexane (final concentration of 50%), acetate (1 mM), and *n*-butylamine (2 M) were added to derivatize acyl-CoA by condensing with butylamide[20]. The reaction was terminated by the addition of an equal amount of 4 M HCl followed by 2 mL of ethyl acetate and was centrifuged at 2,000 × *g* for 10 min. The fatty acid butylamide contained in the ethyl acetate phase was recovered, transferred to a new tube, evaporated under N_2 airflow, and dissolved with hexane.

2.4 Fatty acid analyses

Fatty acid butylamide was analyzed using a gas chromatograph system (GC-2014, Shimadzu, Kyoto, Japan) equipped with a non-polar capillary column (DB-5HT, 0.25 mm × 15 m, Agilent Technology, Santa Clara, CA, USA) under a temperature shift from 100℃ to 380℃ at 0.5℃/

min. γ-Linolenoyl-CoA standard was synthesized by incubating a mixture[40 mM potassium phosphate(pH 7.5), 1 mM ATP, 1 mM $MgCl_2$, 0.2 mM GLA, 1 mM CoA, 0.0025 units/mL acyl-CoA synthetase(Sigma-Aldrich)]at 37℃ for 1 h. The mixture was applied to a silica gel column and, after washing the column with hexane/diethyl ether/acetic acid(3:7:0.1, v/v)to eliminate unreacted γ-linolenic acid, γ-linolenoyl-CoA was eluted with butanol/water/acetic acid (5:3:2, v/v)and derivatized with butylamide as mentioned above to be used as a standard in the gas chromatography analysis.

To analyze fatty acid methyl esters, the yeast cells from 15 mL of culture broth were washed with distilled water and vigorously vortexed in 2 mL of chloroform/methanol (2:1, v/v)plus 0.5 mL of distilled water. The chloroform phase was recovered by centrifugation and methanolysis of total lipid was performed by adding 1 mL of 10% methanolic hydrochloric acid(Tokyo Kasei, Tokyo, Japan)followed by heating at 60℃ for 2 h. After evaporation of the solvents, fatty acid methyl esters(FAMEs)were extracted twice and dissolved in hexane. Fatty acid composition was determined using gas chromatographic system equipped with a capillary column(TC-70, 0.25 mm×30 m, GL Sciences, Tokyo, Japan). FAMEs were identified by comparing their retention time with those of the 37-Component FAME mix(Supelco, Bellefonte, PA, USA). The enzymatic activity of the desaturase expressed in yeast was evaluated using the conversion ratio, which was determined as the ratio of the amount of product to the sum of the amounts of substrate and product and was expressed as a percentage.

2.5 SDS-PAGE and western blotting

The yeast cell homogenate prepared as mentioned in the section 2.3 was centrifuged at 5000×*g* for 10 min and the supernatant was subjected to SDS-PAGE[21]. Proteins separated in the gel were transferred to an Immobilon membrane(Merck Millipore, Darmstadt, Germany)using a semi-dry blotter. The membrane was blocked by immersing in 5% skim milk in PBST(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 0.05%(w/v)Tween-20), and then moved to the same buffer containing mouse anti-FLAG antibody(Sigma-Aldrich; 1:10000). After shaking for 1 h and washing with PBST, the membrane was probed with rabbit anti-mouse IgG(1:20000)for 1 h. The FLAG-tagged proteins were detected by using ECL select(GE Healthcare, Buckinghamshire, UK)and exposure to LAS-500(GE Healthcare, Buckinghamshire, UK).

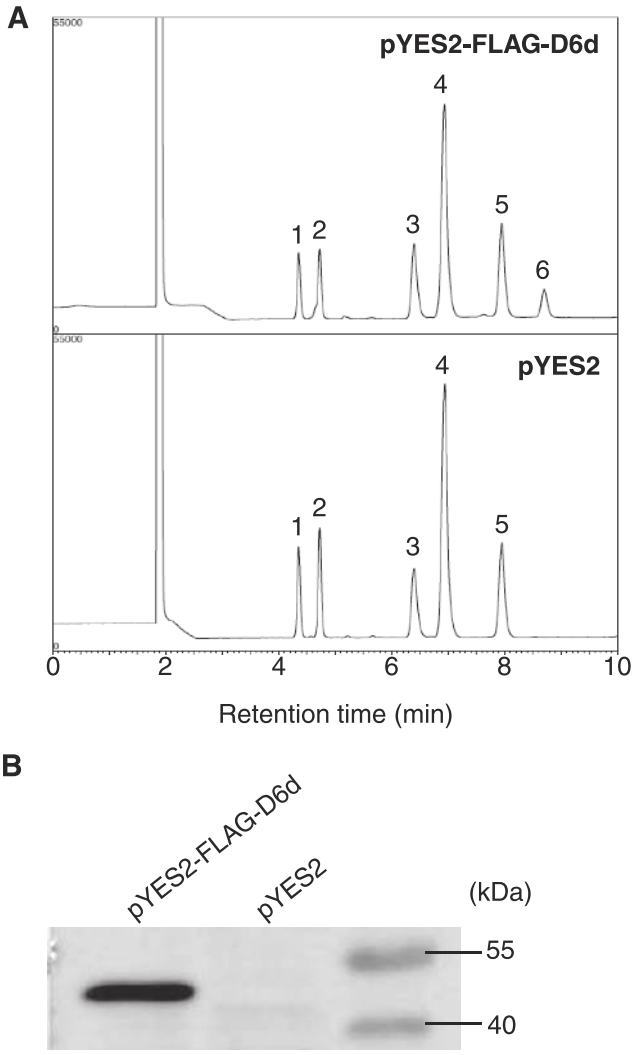

Fig. 1 Expression of active FLAG-D6d in yeast *S. cerevisiae* containing an expression vector, pYES2-FLAG-D6d or pYES2, was cultivated in SCT medium and induced by the addition of galactose. A: Gas chromatograms of fatty acid methyl esters from total lipids in the induced yeast cells. Peak 1, 16:0; 2, 16:1; 3, 18:0; 4, 18:1 Δ9; 5, exogenously added 18:2 Δ9,12; 6, product 18:3 Δ6,9,12. B: Western blot analysis. Total proteins were separated by SDS-PAGE and transferred to a membrane. The FLAG-D6d was detected with anti-FLAG antibody as described in the text.

3 Results

3.1 Functional expression of FLAG-D6d in yeast

The D6d gene tagged with a FLAG peptide at the N-terminal was expressed in *S. cerevisiae* INVSc-1 in the presence of LA, and the intracellular fatty acid composition was determined. As shown in Fig. 1A, GLA, as the Δ6 desaturation product from LA, was generated at the conversion rate of 23.1%. It was not detected at all in the control experiment using an empty vector. The western blotting analysis using anti-FLAG antibody could detect the production of

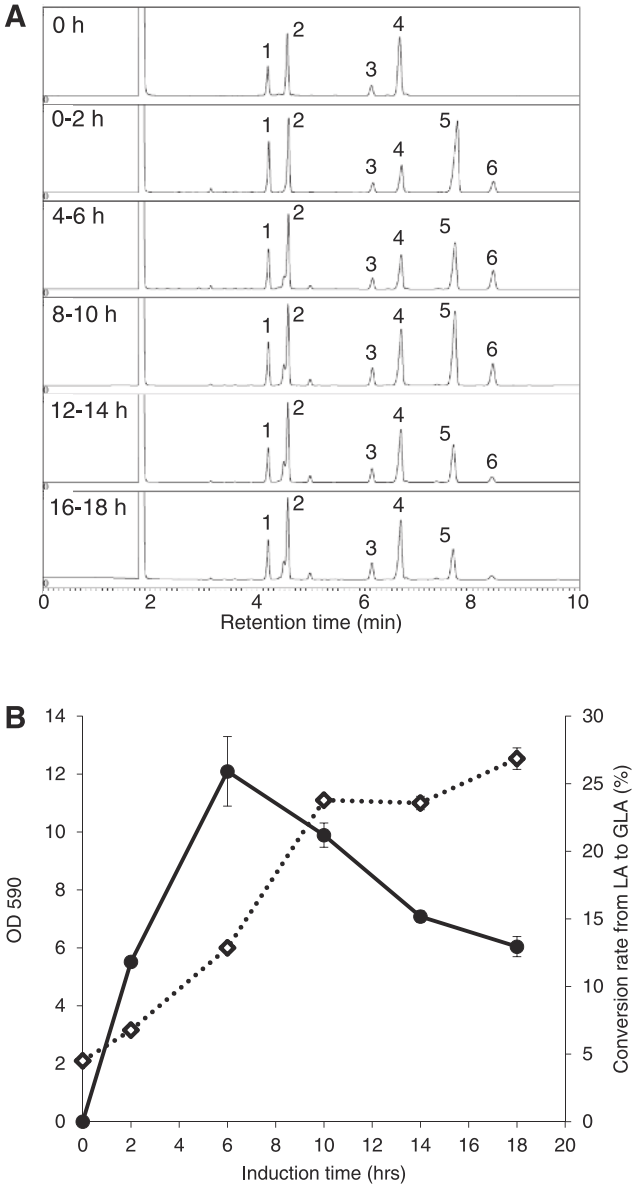

Fig. 2 Time-course of *in vivo* D6d activity in *S. cerevisiae*. The expression of D6d gene was induced by the addition of galactose to the culture medium at 0 h. and LA was added at 0, 4, 8, 12, or 16 h after the induction. The yeast cells were harvested 2 h after the addition of substrate and fatty acid composition in total lipid was analyzed. A: Gas chromatograms of fatty acid methyl esters from total lipids of the D6d-expressed yeast cells harvested just before the addition of substrate (0 h) or at the end of each reaction period (2, 6, 10, 14, or 18 h). Peak numbers are the same as in Fig. 1B: Yeast cell growth (OD; open diamonds) and D6d activity (filled circles). The D6d activity was indicated as conversion rate of LA to GLA 2 h after the addition of substrate.

the 52-kDa FLAG-D6d protein, only when the D6d gene was expressed (Fig. 1B). Therefore, the active D6d with a FLAG tag was successfully expressed in yeast.

3.2 Timing of expression of maximum D6d activity

In the yeast system, the expression of the D6d gene, under the control of Gal1 promoter was stably induced by the addition of galactose. However, the time required to reach the maximum activity of D6d had not been determined. During the cultivation of D6d-expressing yeast cells in the presence of galactose, the substrate, LA, was added at various time points. Two hours after each addition, yeast cells were harvested and their fatty acid composition was measured. The maximum rate of conversion from LA to GLA (25.9%) was observed when LA was added 4-6 hours after induction, as seen in Fig. 2. Thus, 4 h after the gene

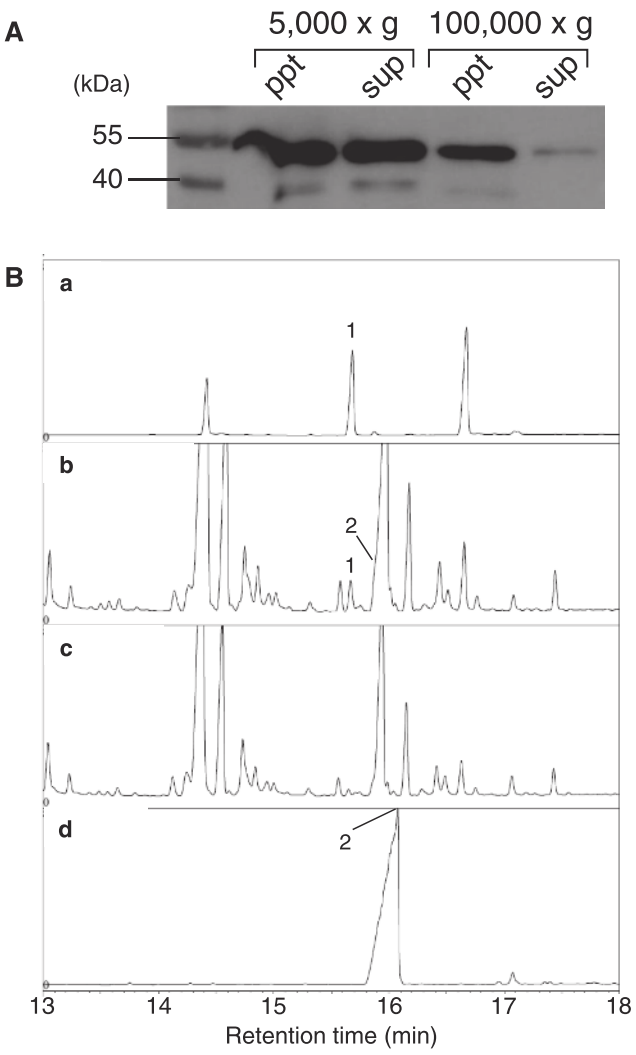

Fig. 3 *In vitro* desaturase reaction by homogenate or microsomes prepared from D6d-expressing yeast cells with linoleoyl-CoA. A: Disrupted yeast cells were centrifuged at 5000 × *g* and the resultant supernatant (sup) was ultracentrifuged at 100,000 × *g* to recover the microsomes (ppt). The FLAG-D6d was detected by western blotting using anti-FLAG antibody. B: *In vitro* desaturation reaction by cell homogenate with linoleoyl-CoA (panel b). After the reaction for 30 min, acyl-CoAs were derivatized with *n*-butylamine and analyzed by gas chromatography. The D6d product was identified by comparing the retention time with that of γ-linolenoyl butylamide (peak 1, panel a) derivatized from γ-linolenoyl-CoA, synthesized as described in Experimental procedures. Panel a: γ-linolenoyl butylamide standard; c: reaction mixture without linoleoyl-CoA; d: reaction mixture without cell homogenate. Peak 1: γ-linolenoyl butylamide; 2: linoleoyl butylamide. Other peaks in panel a are derived from Triton X-100.

induction was identified as the best time to obtain the maximum D6d activity, and was used to harvest the yeast cells for *in vitro* reaction.

3.3 *In vitro* D6d reaction using yeast cell homogenate and microsomes

The D6d-expressing yeast cells were mechanically disrupted, and the microsome fraction was prepared from the homogenate to detect the successful expression of the FLAG-D6d by western blotting as shown in Fig. 3A. The homogenate and microsomes containing the D6d protein were used for *in vitro* desaturase reaction with the substrate, linoleoyl-CoA. Figure 3B shows the gas chromatograms of the butylamide derivatives of the product with the same retention time as γ-linolenoyl butylamide generated in the reaction mixture using the cell homogenate. This result demonstrated the usefulness of the proposed method for *in vitro* desaturation assay. However, this com-

pound was not detected when no substrate was added to the reaction or microsomes were used instead of the homogenate (data not shown).

4 Discussion

Previously reported *in vitro* desaturase reactions, using microsomes as an enzyme fraction and acyl-CoA as a substrate, had to perform saponification and methylesterification of acyl-CoA products to detect them by gas chromatography[7, 8]. Since fatty acids contained in glycerolipids and glycolipids in the membrane fractions are methylesterified as well, the reaction product could not be discriminated from endogenous compounds. The use of radio- and stable isotopes of fatty acid substrate requires a restricted area and exclusive facilities, and is often not quantitative in nature. In this study, non-labeled acyl-CoA was used as a substrate, the reaction product was derivatized with butylamide[20], and detected by gas chromatography (Fig. 3B). To the best of our knowledge, this is the first attempt to detect the *in vitro* desaturation product using non-labeled acyl-CoA as the substrate. Since butylamine reacts only with fatty acid thioesters, but not with esters without thiol, this approach distinguishes fatty acids generated by the desaturation reaction from the endogenous species. The butylamidation treatment after the purification of the generated acyl-CoA[20], if necessary, will diminish the disturbances in the background and fulfill the criteria for quantitative measurement. Moreover, the substrate, acyl-CoA, can be obtained from manufacturers or synthesized by users themselves through chemical procedures[22], and enzymatic methods that have been applied to prepare γ-linolenoyl-CoA in this study. Therefore, any species of fatty acid can be used as a substrate.

In *in vitro* desaturation reaction using membrane fractions from animal tissues[7] and plant plastids[8], the fatty acid substrate and product may be consumed or modified by intrinsic metabolic systems. This might be the case even in the yeast heterologous expression system like the one used in this study as only the cumulative total of the generated product has been measured. Therefore, it would be more precise to determine the change of substrate and product concentrations with the lapse of time during the reaction. Here, we have investigated the time to obtain the maximum D6d activity after the induction of gene expression and determined that 4 h post induction was the best time to harvest the yeast cells for use in *in vitro* reaction. The appropriate timing should be set for each instance of the experiment.

Since the mammalian membrane-bound type desaturases are known to associate with endoplasmic reticulum[23], the D6d activity was expected to be concentrated in the microsome fraction. Indeed, the D6d protein was detected in the precipitates after ultracentrifugation but the generation of γ-linolenoyl-CoA was not observed in *in vitro* reaction with the microsomes (data not shown). In *in vitro* desaturation reactions using microsomes prepared from rat liver or flax seeds, the addition of supernatant obtained after the ultracentrifugation, or the supplementation of purified catalase was effective in boosting the desaturase activity[24, 25]. The identification of such a specific component that results in maximum activity will be critical in standardizing the *in vitro* desaturase reaction.

5 Conclusion

The *in vitro* reaction using desaturase-expressing yeast cell homogenate followed by the butylamidation of the acyl-CoA substrate and product could detect the activity of membrane-bound type desaturase against non-labeled substrate for the first time. This quantitative system with low background expression will contribute toward elucidating the enzymatic machinery of fatty acid desaturases.

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