



Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a

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ABSTRACT

Hydroxy fatty acid dehydrogenase, which is involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a, was cloned, expressed, purified, and characterized. The enzyme preferentially catalyzed NADH-dependent hydrogenation of oxo fatty acids over NAD⁺-dependent dehydrogenation of hydroxy fatty acids. In the dehydrogenation reaction, fatty acids with an internal hydroxy group such as 10-hydroxy-*cis*-12-octadecenoic acid, 12-hydroxy-*cis*-9-octadecenoic acid, and 13-hydroxy-*cis*-9-octadecenoic acid served as better substrates than those with α - or β -hydroxy groups such as 3-hydroxyoctadecanoic acid or 2-hydroxyeicosanoic acid. The apparent K_m value for 10-hydroxy-*cis*-12-octadecenoic acid (HYA) was estimated to be 38 μ M with a k_{cat} of $7.6 \times 10^{-3} \text{ s}^{-1}$. The apparent K_m value for 10-oxo-*cis*-12-octadecenoic acid (KetoA) was estimated to be 1.8 μ M with a k_{cat} of $5.7 \times 10^{-1} \text{ s}^{-1}$. In the hydrogenation reaction of KetoA, both (R)- and (S)-HYA were generated, indicating that the enzyme has low stereoselectivity. This is the first report of a dehydrogenase with a preference for fatty acids with an internal hydroxy group.

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1. Introduction

Functional lipids have attracted attention both nutritionally and pharmaceutically. Conjugated linoleic acid (CLA) is a representative functional fatty acid, which has beneficial effects such as decreasing body fat content [1] and preventing tumorigenesis [2,3] and arteriosclerosis [4]. Oxo fatty acids as well as CLA have also been proven to have novel physiological functions. For example, it has recently been reported that 13-oxo-9,11-octadecadienoic acid in tomato juice acts as a potent peroxisome proliferator activated receptor α (PPAR α) agonist and improves dyslipidemia and hepatic steatosis induced by obesity [5].

In our previous study, we revealed polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a [6], which is a strain with a potential to produce CLA from

linoleic acid [7–10]. The novel saturation metabolism consisted of four enzymes: CLA-HY (hydratase/dehydratase) [6,11,12], CLA-DH (dehydrogenase), CLA-DC (isomerase), and CLA-ER (enone reductase) [6,12]. This saturation metabolism included some oxo fatty acids, such as 10-oxo-*cis*-12-octadecenoic acid (KetoA), 10-oxooctadecanoic acid, and 10-oxo-*trans*-11-octadecenoic acid, as intermediates. These oxo fatty acids are expected to have new physiological activities. CLA-DH generated these oxo fatty acids through dehydrogenation of the corresponding hydroxy fatty acids, e.g., dehydrogenation of HYA to KetoA.

In this study, we describe the enzymatic and physiochemical characteristics of CLA-DH, which is involved in the saturation metabolism and catalyzes the dehydrogenation of hydroxy fatty acids and the hydrogenation of oxo fatty acids.

2. Materials and methods

2.1. Chemicals

HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid, (S)-10-hydroxy-*cis*-12,15-octadecadienoic acid, (S)-10-hydroxy-*cis*-6,12-octadecadienoic acid, and

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13-hydroxy-*cis*-9-octadecenoic acid were prepared as previously described [6,11,13,14]. Oxo fatty acids (KetoA, 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid, 10-oxo-*cis*-12,15-octadecadienoic acid, 10-oxo-*cis*-6,12-octadecadienoic acid, 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid) were prepared from hydroxy fatty acids (HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid, (*S*)-10-hydroxy-*cis*-12,15-octadecadienoic acid, (*S*)-10-hydroxy-*cis*-6,12-octadecadienoic acid, ricinoleic acid, and 13-hydroxy-*cis*-9-octadecenoic acid) by Jones oxidation, which is oxidation of the hydroxy group with CrO_3 [15]. (*R*)-HYA was purified from racemic HYA using HPLC equipped with a chiral column in the same method as “Enantiomeric purity analysis of hydroxy fatty acids” shown in below. Fatty acid-free (<0.02%) bovine serum albumin (BSA) was purchased from Sigma (St. Louis, USA). All other chemicals were of analytical grade and were commercially obtained.

2.2. Preparation of CLA-DH

Escherichia coli Rosetta2/pCLA-DH [12] cells were cultured in 1.5 L of Luria-Bertani (LB) medium at 37 °C for 2 h with simultaneous shaking at 100 rpm, and then isopropyl- β -thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. After adding IPTG, the transformed cells were cultivated at 20 °C for 8 h with simultaneous shaking at 100 rpm. After cultivation, the transformed cells (8 g) were harvested, suspended in a standard buffer (16 mL), and treated with an ultrasonic oscillator (5 min, 4 times, Insinator 201 M; Kubota, Japan). The standard buffer contained 1 mM DTT and 10% (v/v) ethylene glycol in 20 mM potassium phosphate buffer (KPB) (pH 6.5). The cell debris was removed by centrifuging at $1700 \times g$ for 10 min. The resulting supernatant solutions were used as cell-free extracts. The cell-free extracts were fractioned ultracentrifugation at $100,000 \times g$ for 60 min and the supernatant was obtained. CLA-DH was purified from this supernatant using a fast protein liquid chromatography (FPLC) system (GE Healthcare) equilibrated with the standard buffer. The supernatant was applied to a HiLoad 26/60 Superdex 200 prep-grade column (GE Healthcare) that had already been equilibrated with standard buffer and eluted. CLA-DH was further purified using a Mono Q 10/100 GL column (GE Healthcare), a Superdex 200 10/300 GL column (GE Healthcare), and a Phenyl Superose HR 10/10 (Pharmacia). The purified CLA-DH was dialyzed with the standard buffer including 50% (v/v) glycerol and stored at –20 °C until further use.

2.3. Determination of the molecular mass of CLA-DH

In order to determine the native molecular mass of CLA-DH, the enzyme solution was subjected to high performance gel-permeation chromatography on a G-3000SW column (0.75 $\times$ 60 cm, Tosoh, Tokyo, Japan) at room temperature. It was eluted with 100 mM KPB (pH 6.5) containing 100 mM Na_2SO_4 at a flow rate of 0.5 mL/min. The absorbance of the effluent was monitored at 280 nm. The molecular mass of the enzymes was determined from their mobility relative to those of standard proteins.

2.4. Reaction conditions

All operations were performed in an anaerobic chamber. The standard reaction conditions were as described. The reactions were performed in test tubes (16.5 $\times$ 125 mm) that contained 1 mL of reaction mixture (20 mM sodium succinate buffer, pH 4.5) with 0.1% (w/v) HYA or KetoA complexed with BSA [0.02% (w/v)] as the substrate, 5 mM NAD^+ or NADH and 42 μg (=0.04 U/mL) purified

CLA-DH. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 μmol of HYA per minute. The reactions were performed under anaerobic conditions in a sealed chamber with an O_2 -absorbent (Anaeropack “Kenki,” Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan) and gently shaken (120 strokes/min) at 37 °C for 15 min. All experiments were performed in triplicate. Reactions were performed under the standard reaction conditions with some modifications, as described below. The optimal reaction temperature was determined by incubating 1 mL of the reaction mixture (20 mM sodium succinate buffer, pH 4.5) at various temperatures for 15 min under anaerobic conditions. The optimal reaction pH was determined at 37 °C using 1 mL of 20 mM sodium citrate buffer (pH 3.0–4.0) or 20 mM sodium succinate buffer (pH 4.0–5.5). Thermal stability was determined by measuring the enzyme activity after incubating 1 mL of reaction mixture containing 20 mM sodium succinate buffer (pH 4.5) at various temperatures for 15 min under anaerobic conditions. The pH stability was determined by measuring enzyme activity after incubating at 37 °C for 10 min in the following buffers under anaerobic conditions: sodium citrate buffer (50 mM; pH 3.0–4.0), sodium succinate buffer (50 mM; pH 4.0–6.0), KPB (50 mM; pH 5.5–8.0), and Tris-HCl buffer (50 mM; pH 7.0–9.0).

2.5. Kinetic analysis

All procedures were performed in an anaerobic chamber. Reactions were performed under standard reaction conditions with modified substrate and enzyme concentrations. The kinetics of HYA dehydrogenation were studied using 30–1000 μM HYA complexed with 0.02% (w/v) BSA as the substrate, 7 $\mu\text{g/mL}$ CLA-DH, and a reaction time of 15 min. The kinetics of KetoA dehydrogenation were studied using 1–20 μM KetoA complexed with 0.02% (w/v) BSA as the substrate, 0.35 $\mu\text{g/mL}$ CLA-DH, and a reaction time of 5 min. The kinetic parameters were calculated by using the experimental data with the Michaelis–Menten equation using KaleidaGraph 4.0 (Synergy Software Inc., PA, USA).

2.6. Lipid analysis

Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an internal standard. Lipids were extracted from 1 mL of the reaction mixture using 5 mL of chloroform/methanol/1.5% (w/v) KCl in H_2O (2:2:1, by volume) according to the procedure of Bligh–Dyer, and then concentrated by evaporation under reduced pressure [16]. The resulting lipids were dissolved in 5 mL of benzene/methanol (3:2, by volume) and methylated with 300 μL of 1% trimethylsilyldiazomethane (in hexane) at 28 °C for 30 min. After methyl esterification, the resulting fatty acid methyl esters were concentrated by evaporation under reduced pressure. The resulting fatty acid methyl esters were analyzed by gas–liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector, a split injection system, and a capillary column (SPB-1, 30 m $\times$ 0.25 mm I.D., SUPELCO, PA, USA). The initial column temperature 180 °C (for 30 min) was subsequently increased to 210 °C at a rate of 60 °C/min, and then maintained at 210 °C for 29.5 min. The injector and detector were operated at 250 °C. Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The fatty acid peaks were identified by comparing the retention times to those of known standards.

2.7. Enantiomeric purity analysis of hydroxy fatty acids

The enantiomeric purity of HYA, which was produced from KetoA hydrogenation with CLA-DH, was analyzed using HPLC (Shimadzu, Kyoto, Japan) using a Shimadzu LC 20A System (Shimadzu)

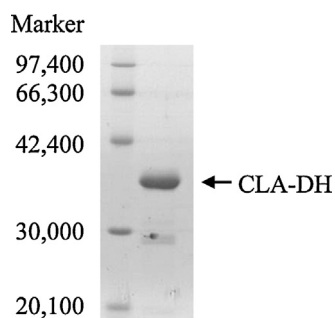


Fig. 1. SDS-PAGE analysis of purified CLA-DH. Molecular mass standards: from the top, phosphorylase b (97,200), bovine serum albumin (66,400), ovalbumin (45,000), carbonic anhydrase (29,000), and trypsin inhibitor (20,100). The observed molecular weight of purified CLA-DH was 40 kDa.

equipped with a chiral column (Chiralpak IA, 4.6 mm I.D., Daicel, Osaka, Japan) and an Evaporative Light Scattering Detector System (Shimadzu, Kyoto, Japan) as a detector. Acetonitrile/0.2% formic acid (65:35) was used as a solvent at a flow rate of 1.0 mL/min. Racemic HYA prepared from KetoA reduction with NaBH₄ [17] was used as the standard.

3. Results

3.1. Purification of CLA-DH

The recombinant CLA-DH without the tag was purified to homogeneity from cell-free extracts of the transformed *E. coli* through four steps of column chromatography. The purified CLA-DH displayed a single band on an SDS-PAGE gel (Fig. 1). The observed molecular mass of the subunit was 40 kDa, corresponding to a calculated mass of 32 kDa deduced from the amino acid sequence of its gene. The relative native molecular mass was estimated to be 32 kDa by HPLC on a G-3000SW column, indicating that the enzyme consists of the single subunit. The purified CLA-DH was used for further characterization.

3.2. Effects of reaction conditions

CLA-DH required NAD⁺/NADH as a cofactor but not NADP⁺/NADPH. The effects of NAD⁺/NADH concentration were examined from 0 to 7.5 mM (Fig. 2a). The dehydrogenation and hydrogenation activities increased with increasing concentrations of NAD⁺/NADH. The effects of temperature were also examined. The optimal reaction temperature was found to be 52 °C (Fig. 2b). The effects of pH were examined over a pH range from 3.0 to 5.5 with an optimal reaction pH determined to be pH 4.5 (Fig. 2c).

3.3. Enzyme stability

The thermal stability of the purified enzyme was investigated from 18 °C to 67 °C. The enzyme was incubated at each temperature for 15 min at pH 4.5. More than 80% of the initial activity remained at temperatures up to 28 °C (Fig. 3a). The pH stability of the purified enzyme was investigated by incubating the enzyme in different buffers within a pH range of 3.0 to 9.0 for 10 min at 37 °C. More than 80% of the initial activity remained in a pH range from 4.5 to 7.5 (Fig. 3b).

3.4. Substrate specificity

In the dehydrogenation reaction, 10-, 12-, or 13-hydroxy C18 fatty acids such as HYA, (R)-HYA, 10-hydroxyoctadecanoic

Table 1

Substrate specificity of CLA-DH for dehydrogenation.

Substrate	Relative activity [%]
(S)-10-Hydroxy- <i>cis</i> -12-octadecenoic acid (HYA)	100 ^a
(R)-10-Hydroxy- <i>cis</i> -12-octadecenoic acid ((R)-HYA)	98
10-Hydroxyoctadecanoic acid	25
10-Hydroxy- <i>trans</i> -11-octadecenoic acid	69
(S)-10-Hydroxy- <i>cis</i> -12, <i>cis</i> -15-octadecadienoic acid	75
(S)-10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	54
(R)-12-Hydroxy- <i>cis</i> -9-octadecenoic acid	62
13-Hydroxy- <i>cis</i> -9-octadecenoic acid	142
3-Hydroxyoctadecanoic acid (C18)	– ^b
3-Hydroxytetradecanoic acid (C14)	–
2-Hydroxyeicosanoic acid (C20)	–
Methyl (S)-10-Hydroxy- <i>cis</i> -12-octadecenoate	127
8-Hexadecanol	24

^a The activity of (S)-10-hydroxy-*cis*-12-octadecenoic acid dehydrogenation (=0.048 U/mg) under the condition (5 mM NAD⁺; 37 °C, pH 4.5, 15 min) was defined as 100%.

^b –, Not detected.

Table 2

Substrate specificity of CLA-DH for hydrogenation.

Substrate	Relative activity [%]
10-Oxo- <i>cis</i> -12-octadecenoic acid (KetoA)	100 ^a
10-Oxo-octadecanoic acid	66
10-Oxo- <i>trans</i> -11-octadecenoic acid	53
10-Oxo- <i>cis</i> -12, <i>cis</i> -15-octadecadienoic acid	44
10-Oxo- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	6
12-Oxo- <i>cis</i> -9-octadecenoic acid	87
13-Oxo- <i>cis</i> -9-octadecenoic acid	156
Methyl 10-oxo- <i>cis</i> -12-octadecenoate	170
7-Hexadecanone	332

^a The activity of 10-oxo-*cis*-12-octadecenoic acid hydrogenation (=0.22 U/mg) under the condition (5 mM NADH; 37 °C, pH 4.5, 15 min) was defined as 100%.

acid, 10-hydroxy-*trans*-11-octadecenoic acid, (S)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid, (S)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, (R)-12-hydroxy-*cis*-9-octadecenoic acid, and 13-hydroxy-*cis*-9-octadecenoic acid served as good substrates and transformed into corresponding 10-, 12-, or 13-oxo fatty acids. In addition, HYA methyl ester and 8-hexadecanol were dehydrogenated to KetoA methyl ester and 8-hexadecanone, respectively. In contrast, 2- or 3-hydroxy fatty acids such as 3-hydroxyoctadecanoic acid, 3-hydroxytetradecanoic acid, and 2-hydroxyeicosanoic acid were not dehydrogenated (Table 1).

In the hydrogenation reaction, 10-, 12- or 13-oxo C18 fatty acids such as KetoA, 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid, 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid, 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid served as good substrates and transformed into corresponding 10-, 12- or 13-hydroxy fatty acids. In addition, KetoA methyl ester and 7-hexadecanone were hydrogenated to HYA methyl ester and 7-hexadecanol, respectively (Table 2).

3.5. Kinetic analysis of the CLA-DH catalyzing reactions

The substrate concentration-reaction velocity curves for HYA dehydrogenation and KetoA hydrogenation were used with the Michaelis–Menten equation. The apparent K_m value for HYA in the dehydrogenation reaction was estimated to be 38 μM with a k_{cat} of $7.6 \times 10^{-3} \text{ s}^{-1}$. The apparent K_m value for KetoA in the hydrogenation reaction was estimated to be 1.8 μM with a k_{cat} of $5.7 \times 10^{-1} \text{ s}^{-1}$.

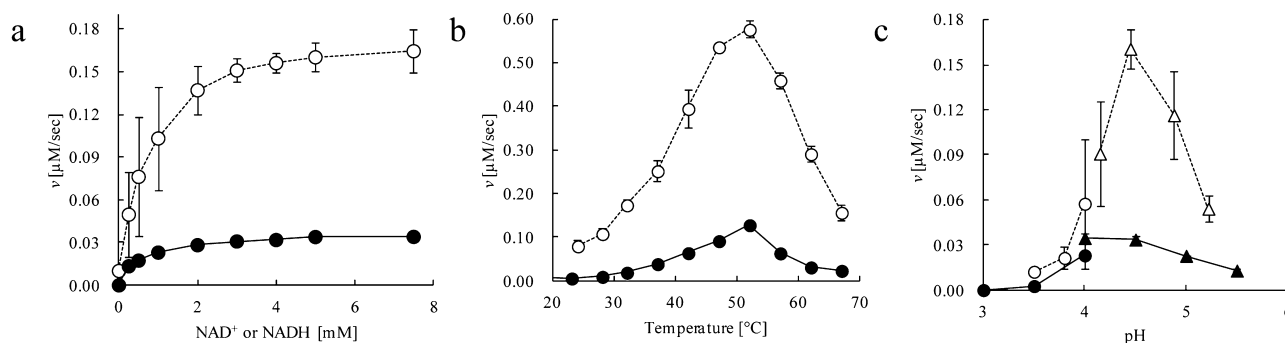


Fig. 2. Effects of NAD^+/NADH concentrations, temperature, and pH on the activity of CLA-DH. (a) Effects of NAD^+/NADH concentrations: dehydrogenation activity (closed circles) and hydrogenation activity (open circles) were assayed under standard reaction conditions, except for NAD^+/NADH concentrations. (b) Effects of temperature: dehydrogenation activity (closed circles) and hydrogenation activity (open circles) were assayed under standard reaction conditions, except for the temperature. (c) Effects of pH: activity was assayed under standard reaction conditions, except for the buffers used. Sodium citrate buffer (closed and open circles for dehydrogenation and hydrogenation, respectively), pH 3.0–4.0, and sodium succinate buffer (closed and open triangles for dehydrogenation and hydrogenation, respectively), pH 4.0–5.5, were used.

3.6. Enantiomeric purities of the hydroxy fatty acids produced by CLA-DH

The enantiomeric purity of HYA produced from KetoA by CLA-DH was analyzed using HPLC with a chiral column. Almost the same amounts of both enantiomers of (R)-HYA and (S)-HYA were produced from KetoA, indicating that CLA-DH had low stereoselectivity in oxo fatty acid hydrogenation.

3.7. Effects of chemicals on the enzyme activity

The effects of metal ions and inhibitors (1 mM) were investigated in both the hydration and dehydration reactions. The reactions were strongly inhibited by Ag^+ , Cu^{2+} , Hg^{2+} , VO_3^- , WO_4^{2-} , and aluminon (data not shown). 2,3,5-Triphenyltetrazolium inhibited only dehydrogenation activity.

4. Discussion

We revealed polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU 1009a and identified CLA-DH. The CLA-DH gene was located together with CLA-DC (fatty acid isomerase) and CLA-ER (fatty acid enone reductase) genes involved in polyunsaturated

fatty acid saturation metabolism in *L. plantarum* AKU 1009a [6]. These results suggested that CLA-DH plays an important role in saturation metabolism.

CLA-DH, which belongs to the short-chain dehydrogenase/reductase (SDR) family, showed considerable similarity with other SDRs (Fig. 4). In this paper, we characterized CLA-DH from the aspect of its physiological function to clarify its distinct characteristic properties in the SDR family, especially from the viewpoint of substrate specificity. There are few reports regarding either hydroxy fatty acid dehydrogenation or oxo fatty acid hydrogenation in the SDR family. However, CLA-DH catalyzed the dehydrogenation or hydrogenation of fatty acids which have an internal hydroxy or an oxo group, respectively (Tables 1 and 2). *Micrococcus luteus* WIUJH-20 was reported to convert 10- or 12-hydroxyoctadecanoic acid to the corresponding oxooctadecanoic acid. The amino acid sequence of the enzyme which catalyzes the above oxidation of hydroxy fatty acid in *M. luteus* WIUJH-20 belongs to a secondary alcohol dehydrogenase [18]. The amino acid sequence of the secondary alcohol dehydrogenase from *M. luteus* did not resemble that of CLA-DH, indicating that the dehydrogenation activity of CLA-DH was characteristic activity among the SDR family.

As a characteristic property of CLA-DH, the enzyme showed higher activity in hydrogenation than dehydrogenation reactions.

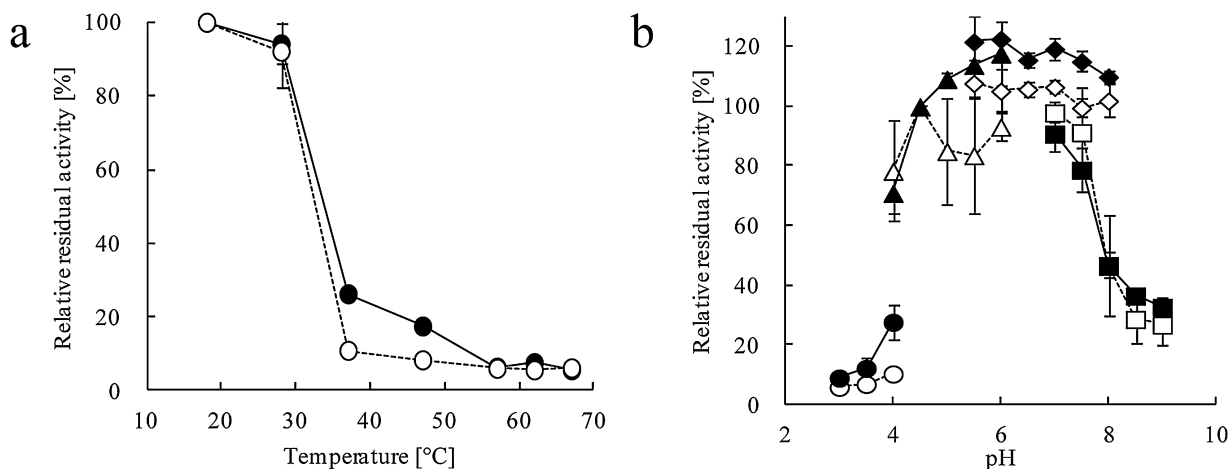


Fig. 3. Effects of temperature and pH on stability of CLA-DH. (a) Effect of temperature: the thermal stability of the dehydrogenation activity (closed circles) and hydrogenation activity (open circles) were assessed under standard reaction conditions after incubation at each temperature (18–67 °C) for 30 min. The activities after incubation at 18 °C were defined as 100% for dehydrogenation (0.048 U/mg) and hydrogenation (0.22 U/mg). (b) Effect of pH: the pH stabilities of the dehydrogenation (closed) and hydrogenation (open) reactions were evaluated under standard reaction conditions after incubation at 37 °C for 10 min at each pH. Sodium citrate buffer, pH 3.0–4.0 (circles), sodium succinate buffer, pH 4.0–6.0 (triangles), potassium phosphate buffer, pH 5.0–7.5 (diamonds), and Tris-HCl buffer, 7.0–9.0 (squares) were used. The activities after incubation in sodium succinate buffer (pH 4.5) were defined as 100% for dehydrogenation (0.042 U/mg) and hydrogenation (0.22 U/mg).

CLA-DH	1	--MKDFKDKVMFITGAAHGFQGVIAEGAADRGMKLTIVDIDEPALKKTYQHILDKGAEVL	58
AADH	1	-MFNSIEGRSVVVTGGSKGIGLGMVRV FARAGANV LMTARDALT LERAAEGLNGLPGAVS	59
LbRADH	1	-MSNRLDGKVAIVTGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEKAASVGTDPDQIQF	59
TtADH	1	MGLF--AGKGVLV TGGARGIGRAIAQAFAREGALVALCDLRPEGKE--VAEAIGGAFFQV	56
LSADH	1	MAQYDVADRS AIVTGGGSGIGRAVALTLAASGA AVLVTDLNEEHAQAVVAEIEAAGGKAA	60
TGXXXGXG			
CLA-DH	59	MVTADV TKEASVDDAVEQAMEKFGRIDL LINNAGI ALP-GRIWELPTRDWEWIMHINLMS	117
AADH	60	TLQVDVTNPDSLAGMAEVAAERHGGIDVLCANAGIFPS-KRLGEMTSEDMDSVFGVNVKG	118
LbRADH	60	FQHDSSDED-GWTKLFDATEKAFGPVSTLVNNAGIAVN-KSVEETTTAEWRKLLAVNLDG	117
TtADH	57	DLEDERERV-RFVEE---AAYALGRVDVLVNNAIAAPGSAL-TVRLPEWRRRVLEVNLTA	111
LSADH	61	ALAGDVTDP-AFGEASVAGANALAPLKI AVNNAGIGGEAATVGDYSLDSWRTVIEVNLNA	119
*			
CLA-DH	118	QVYAMKRVIPIMIQQKTHADILNVA SIAGLVDT-PGMPSYHASKFASVGMTEATAYDLQR	176
AADH	119	TIHAVQACMPWLETSGRGRVVVTS-SITGPVTGYPGWSHYGASKAAQMGFIRTA AIELAP	177
LbRADH	118	VFFGTRLGIQRMKNKGLGASIIINMSSIEGFV-GDPSLGAYNASKGAVRIMSKSAALDCAL	176
TtADH	112	PMHLSALAAREMRKVG-GGAIVNVASVQGLF-AEQENAAYNASKGGLVNLTRSLALDLAP	169
LSADH	120	VFYGMQPQLKAMAANG-GGAIVNMA SI LGSV-GFANSSAYVTAKHALLGLTQNAALEYAA	177
* * *			
CLA-DH	177	ANIDIDMHVMCPGFVQTDLYHTENHRPAQYS DPTDPYYQSEAYLKGQQFAKYVITNGKPI	236
AADH	178	KR--ITINAVLPGNVITEG---LDGLGQEY---LDQMASSVPAG-----SLGSVE	219
LbRADH	177	KDYDVRVNTVHPGYIKTP--LVDDLPGAE---EAMSQRTKTPMG-----HIGEPN	221
TtADH	170	LR--IRVNAVAPGAIATEAVLEAIALSPDPERTRRDWEDLHALR-----RLGKPE	217
LSADH	178	DK--VRVVAVGPGFIRTP--LVEANLSAD---ALAFLEGKHALG-----RLGEPE	220
CLA-DH	237	DTIADTVFKALEDNRFYILTHPEYNPLIEDRVKRIVTDGAPDVHIMDGIM---	286
AADH	220	DIANAALFFALDEAAAYITG-----QSLIVDGGQVLPESAMALGEL	259
LbRADH	222	DIAYICVYLASNESKFATG-----SEFVVDGGGYTAQ-----	252
TtADH	218	EVAEAVLFLASEKASFITG-----AILPVDGGMTASFMMAGR PV-	256
LSADH	221	EVASLVAFLASDAASFITG-----SYHLVDGGGYTAQ-----	251

Fig. 4. Multiple-sequence alignment of CLA-DH and ADHs belonging to the SDR family. The SDR family includes *Rhodococcus erythropolis* (AADH), *Lactobacillus brevis* (LbRADH), *Thermus thermophilus* (TtADH), and *Leifsonia* sp. strain S749 (LSADH). The accession numbers of the listed proteins are as follows: CLA-DH, BAL42247; AADH, BAF43657; LbRADH, YP.794544; TtADH, YP.003977; LSADH, BAD99642. Black and gray shading indicate residues highly conserved in the SDR family. TGXXXGXG is co-enzyme binding region in typical SDRs. The star indicates the four members of catalytic tetrad.

The activity of KetoA hydrogenation was 5 times higher than that of HYA dehydrogenation (Fig. 2).

Although many SDRs have high enantioselectivity [19–23], CLA-DH had low enantioselectivity to dehydrogenate both (*R*) and (*S*) hydroxy fatty acids (Table 1) and produce (*R*) and (*S*) hydroxy fatty acids from oxo fatty acid. In addition, CLA-DH dehydrogenated 10-, 12-, and 13-hydroxy fatty acids (Table 1), indicating its low regioselectivity.

In our previous study, we reported the production of many kinds of hydroxy fatty acids such as 10- and 13-hydroxy octadecapolyenoic acid [6,11,13,14]. Using these various hydroxy fatty acids and hydroxy fatty acid dehydrogenase such as CLA-DH, we can provide many kinds of corresponding oxo fatty acids by applying the wide substrate specificity of CLA-DH. These results enable us to provide new functional lipids, oxo fatty acids.

5. Conclusions

The properties of CLA-HY, a novel hydroxy fatty acid dehydrogenase from *L. plantarum* were investigated. CLA-DH showed wide substrate specificity toward hydroxy fatty acids with a preference to those with an internal hydroxy group. Such substrate preference explained well that CLA-DH is involved in polyunsaturated fatty acid saturation metabolism. From an application oriented

perspective, CLA-DH is useful for the production of oxo fatty acids with unique physiological functions in combination with fatty acid hydratases such as CLA-HY (11, 13), which were reported as good catalysts generating hydroxy fatty acids from common C18 fatty acids.

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