TITLE:

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

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CITATION:

Takeuchi, Michiki ...[et al]. Characterization of hydroxy fatty acid dehydrogenase involved in polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a. *Journal of Molecular Catalysis B: Enzymatic* 2015, 117: 7-12

ISSUE DATE:

2015-04-06

URL:

<http://hdl.handle.net/2433/198740>

RIGHT:

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

3 Running title: Hydroxy fatty acid dehydrogenase from *L. plantarum*

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16

17 **ABSTRACT**

18 Hydroxy fatty acid dehydrogenase, which is involved in polyunsaturated fatty acid
19 saturation metabolism in *Lactobacillus plantarum* AKU 1009a, was cloned, expressed,
20 purified, and characterized. The enzyme preferentially catalyzed NADH-dependent
21 hydrogenation of oxo fatty acids over NAD⁺-dependent dehydrogenation of hydroxy
22 fatty acids. In the dehydrogenation reaction, fatty acids with an internal hydroxy group
23 such as 10-hydroxy-*cis*-12-octadecenoic acid, 12-hydroxy-*cis*-9-octadecenoic acid, and
24 13-hydroxy-*cis*-9-octadecenoic acid served as better substrates than those with α - or
25 β -hydroxy groups such as 3-hydroxyoctadecanoic acid or 2-hydroxyeicosanoic acid.
26 The apparent K_m value for 10-hydroxy-*cis*-12-octadecenoic acid (HYA) was estimated
27 to be 38 μM with a k_{cat} of $7.6 \cdot 10^{-3} \text{ s}^{-1}$. The apparent K_m value for
28 10-oxo-*cis*-12-octadecenoic acid (KetoA) was estimated to be 1.8 μM with a k_{cat} of
29 $5.7 \cdot 10^{-1} \text{ s}^{-1}$. In the hydrogenation reaction of KetoA, both (*R*)- and (*S*)- HYA were
30 generated, indicating that the enzyme has low stereoselectivity. This is the first report of
31 a dehydrogenase with a preference for fatty acids with an internal hydroxy group.

32 **Keywords:** Lactic acid bacteria; Hydroxy fatty acid; Oxo fatty acid; short-chain
33 dehydrogenase/reductase

34

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

53 hydroxy fatty acids, *e.g.*, dehydrogenation of HYA to KetoA.

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

57

58 2. Materials and methods

59 2.1. Chemicals

60 HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid,
61 (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid,
62 (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, and 13-hydroxy-*cis*-9-octadecenoic
63 acid were prepared as previously described [6,11,13,14]. Oxo fatty acids (KetoA,
64 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid,
65 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid,
66 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid) were prepared
67 from hydroxy fatty acids (HYA, 10-hydroxyoctadecanoic acid,
68 10-hydroxy-*trans*-11-octadecenoic acid, (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic
69 acid, (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, ricinoleic acid, and
70 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 1,700g for 10 min. The resulting supernatant solutions were used as cell-free extracts. The cell-free extracts were fractioned ultracentrifugation at 100,000g 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 (0.75×60 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

107 All operations were performed in an anaerobic chamber. The standard reaction
108 conditions were as described. The reactions were performed in test tubes (16.5×125
109 mm) that contained 1 mL of reaction mixture (20 mM sodium succinate buffer, pH 4.5)
110 with 0.1% (w/v) HYA or KetoA complexed with BSA [0.02% (w/v)] as the substrate, 5
111 mM NAD^+ or NADH and 42 μg (= 0.04 U/ml) purified CLA-DH. One unit was defined
112 as the amount of enzyme that catalyzes the conversion of 1 μmol of HYA per minute.
113 The reactions were performed under anaerobic conditions in a sealed chamber with an
114 O_2 -absorbent (Anaeropack “Kenki,” Mitsubishi Gas Chemical Co., Ltd., Tokyo, Japan)
115 and gently shaken (120 strokes/min) at 37°C for 15 min. All experiments were
116 performed in triplicate. Reactions were performed under the standard reaction
117 conditions with some modifications, as described below. The optimal reaction
118 temperature was determined by incubating 1 mL of the reaction mixture (20 mM
119 sodium succinate buffer, pH 4.5) at various temperatures for 15 min under anaerobic
120 conditions. The optimal reaction pH was determined at 37°C using 1 mL of 20 mM
121 sodium citrate buffer (pH 3.0–4.0) or 20 mM sodium succinate buffer (pH 4.0–5.5).
122 Thermal stability was determined by measuring the enzyme activity after incubating 1
123 mL of reaction mixture containing 20 mM sodium succinate buffer (pH 4.5) at various
124 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 μ M HYA complexed with 0.02% (w/v) BSA as the substrate, 7 μ g/mL CLA-DH, and a reaction time of 15 min. The kinetics of KetoA dehydrogenation were studied using 1–20 μ M KetoA complexed with 0.02% (w/v) BSA as the substrate, 0.35 μ 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 H₂O (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 μ L 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) 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 NaBH_4 [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 $\text{NADP}^+/\text{NADPH}$. 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

197 HYA, (*R*)-HYA, 10-hydroxyoctadecanoic acid, 10-hydroxy-*trans*-11-octadecenoic acid,
198 (*S*)-10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid,
199 (*S*)-10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, (*R*)-12-hydroxy-*cis*-9-octadecenoic
200 acid, and 13-hydroxy-*cis*-9-octadecenoic acid served as good substrates and transformed
201 into corresponding 10-, 12-, or 13-oxo fatty acids. In addition, HYA methyl ester and
202 8-hexadecanol were dehydrogenated to KetoA methyl ester and 8-hexadecanone,
203 respectively. In contrast, 2- or 3-hydroxy fatty acids such as 3-hydroxyoctadecanoic
204 acid, 3-hydroxytetradecanoic acid, and 2-hydroxyeicosanoic acid were not
205 dehydrogenated (Table 1).

206 In the hydrogenation reaction, 10-, 12- or 13-oxo C18 fatty acids such as KetoA,
207 10-oxooctadecanoic acid, 10-oxo-*trans*-11-octadecenoic acid,
208 10-oxo-*cis*-12,*cis*-15-octadecadienoic acid, 10-oxo-*cis*-6,*cis*-12-octadecadienoic acid,
209 12-oxo-*cis*-9-octadecenoic acid, and 13-oxo-*cis*-9-octadecenoic acid served as good
210 substrates and transformed into corresponding 10-, 12- or 13-hydroxy fatty acids. In
211 addition, KetoA methyl ester and 7-hexadecanone were hydrogenated to HYA methyl
212 ester and 7-hexadecanol, respectively (Table 2).

213

214 3.5. Kinetic analysis of the CLA-DH catalyzing reactions

215 The substrate concentration-reaction velocity curves for HYA dehydrogenation and
216 KetoA hydrogenation were used with the Michaelis–Menten equation. The apparent K_m
217 value for HYA in the dehydrogenation reaction was estimated to be 38 μM with a k_{cat} of
218 $7.6 \cdot 10^{-3} \text{ sec}^{-1}$. The apparent K_m value for KetoA in the hydrogenation reaction was
219 estimated to be 1.8 μM with a k_{cat} of $5.7 \cdot 10^{-1} \text{ sec}^{-1}$.

220

221 **3.6. Enantiomeric purities of the hydroxy fatty acids produced by CLA-DH**

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

226

227 **3.7. Effects of chemicals on the enzyme activity**

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

232

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 (Table 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. 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 (Table1) 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

269 The properties of CLA-HY, a novel hydroxy fatty acid dehydrogenase from *L.*
270 *plantarum* were investigated. CLA-DH showed wide substrate specificity toward
271 hydroxy fatty acids with a preference to those with an internal hydroxy group. Such
272 substrate preference explained well that CLA-DH is involved in polyunsaturated fatty
273 acid saturation metabolism. From an application oriented perspective, CLA-DH is
274 useful for the production of oxo fatty acids with unique physiological functions in
275 combination with fatty acid hydratases such as CLA-HY (11, 13), which were reported
276 as good catalysts generating hydroxy fatty acids from common C18 fatty acids.

277

278 **ACKNOWLEDGMENTS**

279 This work was supported, in part, by the Industrial Technology Research Grant Program
280 in 2007 (Grant 07A08005a to S.K.); the Project for the Development of a Technological
281 Infrastructure for Industrial Bioprocesses on Research and Development of New
282 Industrial Science and Technology Frontiers (S.S.) from the New Energy and Industrial
283 Technology Development Organization (NEDO) of Japan; Scientific Research Grants in
284 Aid 19780056 (to S.K.), 16688004 (to J.O.), and 18208009 (to S.S.); the Centers of
285 Excellence for Microbial-Process Development Pioneering Future Production Systems
286 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; the

287 Bio-Oriented Technology Research Advancement Institution of Japan (J.O.); Science
288 and Technology Promotion Program for Agriculture Forestry, Fisheries and Food
289 Industry from the Ministry of Agriculture, Forestry and Fisheries of Japan (J.O.);
290 Advanced Low Carbon Technology Research and Development Program of Japan
291 (S.K.); M.T. and S.K. received Research Fellowship from the Japan Society for the
292 Promotion of Science for Young Scientists.

293

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Figure legends

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.

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.

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°C –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).

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.

376 TGXXXGXG is co-enzyme binding region in typical SDRs. The star indicates the four
377 members of catalytic tetrad.
378

Fig. 1 SDS-PAGE analysis of purified CLA-DH

Fig. 2 Effects of NAD⁺/NADH concentrations, temperature, and pH on the activity of CLA-DH

Fig. 3 Effects of temperature and pH on stability of CLA-DH

CLA-DH	1	--MKDFKDKVMFITGAAHGFGQVIAEGAADRGMKLTIVDIDEPALKKTYQHILDKGAEVL	58
AADH	1	-MFNSIEGRSVVVTGGSGKIGLGMVRFARAGANVLMTARDALTLERAAEGLNGLPGAVS	59
LbRADH	1	-MSNRLDGKVAIVTGGTGLGIGLAIATKFVEEGAKVMITGRHSDVGEKAASVGPDPQIQF	59
TtADH	1	MGLF--AGKGVLTGGARGIGRAIAQAFAREGALVALCDLRPEGKE--VAEAIGGAFFQV	56
LSADH	1	MAQYDVADRSIAIVTGGSGIGRAVALTLAASGA AVLVTDLNEEHAQAVVAEIEAAGGKAA	60
TGXXXGXG			
CLA-DH	59	MVTADVTKESVDDAVEQAMEKFGRIDLLINNAGIALP-GRIWELPTRDWEWIMHINLMS	117
AADH	60	TLQVDVTNPDSLAGMAEVAAERHGGIDVLCANAGIFPS-KRLGEMTSEDMSVFGVNVKG	118
LbRADH	60	FQHDSSDED-GWTKLFDATEKAFGPVSTLVNNAGIAVN-KSVEETTTAEWRKLLAVNLDG	117
TtADH	57	DLEDERERV-RFVEE---AAYALGRVDVLVNNAAIAAPGSAL-TVRLPEWRRVLEVNLT	111
LSADH	61	ALAGDVTDP-AFGEASVAGANALAPLKIAVNNAGIGGEAATVGDYSLDSWRTVIEVNLT	119
*			
CLA-DH	118	QVYAMKRVIPIMIQQKTHADILNVASTAGLVDT-PGMPSYHASKFASVGMTEATAYDLQR	176
AADH	119	TIHAVQACMPWLETSGRGRVVVTS-SITGFPVTGYPGWSHYGASKAAQMGFIRTAIELAP	177
LbRADH	118	VFFGTRLGIQRMKNKGLGASIIINMSSIEGFFV-GDPSLGAYNASKGAVRIMSKSAALDCAL	176
TtADH	112	PMHLSALAAAREMRKVG-GGAIVNVASVQGLF-AEQENAAYNASKGGLVNLTRSLALDLAP	169
LSADH	120	VFYGMQPOLKAMAANG-GGAIVNMASTILGVS-GFANSSAYVTAKHALLGLTQNAALEYAA	177
* * *			
CLA-DH	177	ANIDIDMHVMCPGFVQTDLYHTENHRPAQYSDPTDPYYQSEAYLKGQQFAKYVITNGKPI	236
AADH	178	KR--ITINAVLPGNVITEG---LDGLGQEY---LDQMASSVPAG-----SLGSVE	219
LbRADH	177	KDYDVRVNTVHPGYIKTP--LVDDLPGAE---EAMSQRTKTTPMG-----HIGEPN	221
TtADH	170	LR--IRVNAVPGAIATEAVLEAIALSPDPERTRRDWEDLHALR-----RLGKPE	217
LSADH	178	DK--VRVAVGPGFIRTP--LVEANLSAD---ALAFLEGKHALG-----RLGEPE	220
* * *			
CLA-DH	237	DTIADTVFKALEDNRFYILTHPEYNPLIEDRVKRIVTDGAPDVHIMDGIM---	286
AADH	220	DIANAALFEALDEAAYITG-----QSLIVDGGQVLPESAMALGEL	259
LbRADH	222	DIAYICVYLASNESKFATG-----SEFVVDGGYTAQ-----	252
TtADH	218	EVAEAVLFLASEKASFITG-----AILPVDGGMTASFMMAGRPFV-	256
LSADH	221	EVASLVAFLASDAASFITG-----SYHLVDGGYTAQ-----	251

Fig. 4 Multiple-sequence alignment of CLA-DH and ADHs belonging to the SDR family

Table 1 Substrate specificity of CLA-DH for dehydrogenation.

Substrate	Relative activity [%]
(<i>S</i>)-10-Hydroxy- <i>cis</i> -12-octadecenoic acid (HYA)	100 ^a
(<i>R</i>)-10-Hydroxy- <i>cis</i> -12-octadecenoic acid ((<i>R</i>)-HYA)	98
10-Hydroxyoctadecanoic acid	25
10-Hydroxy- <i>trans</i> -11-octadecenoic acid	69
(<i>S</i>)-10-Hydroxy- <i>cis</i> -12, <i>cis</i> -15-octadecadienoic acid	75
(<i>S</i>)-10-Hydroxy- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	54
(<i>R</i>)-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 (<i>S</i>)-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.

414 Table 2 **Substrate specificity of CLA-DH for hydrogenation.**

415	Substrate	Relative activity [%]
416	10-Oxo- <i>cis</i> -12-octadecenoic acid (KetoA)	100 ^a
417	10-Oxo-octadecanoic acid	66
418	10-Oxo- <i>trans</i> -11-octadecenoic acid	53
419	10-Oxo- <i>cis</i> -12, <i>cis</i> -15-octadecadienoic acid	44
420	10-Oxo- <i>cis</i> -6, <i>cis</i> -12-octadecadienoic acid	6
421	12-Oxo- <i>cis</i> -9-octadecenoic acid	87
422	13-Oxo- <i>cis</i> -9-octadecenoic acid	156
423	Methyl 10-oxo- <i>cis</i> -12-octadecenoate	170
424	7-Hexadecanone	332

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

427