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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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 μM with a k_{cat} of $7.6 \cdot 10^{-3}$ s^{-1}. The apparent K_m value for 10-oxo-*cis*-12-octadecenoic acid (KetoA) was estimated to be 1.8 μM with a k_{cat} of $5.7 \cdot 10^{-1}$ 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.

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 (PPARα) 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

4

71 hydroxy group with CrO_3 [15]. (*R*)-HYA was purified from racemic HYA using HPLC

72 equipped with a chiral column in the same method as "Enantiomeric purity analysis of

73 hydroxy fatty acids" shown in below. Fatty acid-free (<0.02%) bovine serum albumin

74 (BSA) was purchased from Sigma (St. Louis, USA). All other chemicals were of

75 analytical grade and were commercially obtained.

76

77 **2.2. Preparation of CLA-DH**

78 *Escherichia coli* Rosetta2/pCLA-DH [12] cells were cultured in 1.5 L of

79 Luria-Bertani (LB) medium at 37°C for 2 h with simultaneous shaking at 100 rpm, and

80 then isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0

81 mM. After adding IPTG, the transformed cells were cultivated at 20°C for 8 h with

82 simultaneous shaking at 100 rpm. After cultivation, the transformed cells (8 g) were

83 harvested, suspended in a standard buffer (16 mL), and treated with an ultrasonic

84 oscillator (5 min, 4 times, Insinator 201 M; Kubota, Japan). The standard buffer

85 contained 1 mM DTT and 10% (v/v) ethylene glycol in 20 mM potassium phosphate

86 buffer (KPB) (pH 6.5). The cell debris was removed by centrifuging at 1,700*g* for 10

87 min. The resulting supernatant solutions were used as cell-free extracts. The cell-free

88 extracts were fractioned ultracentrifugation at 100,000*g* for 60 min and the supernatant

89   was obtained. CLA-DH was purified from this supernatant using a fast protein liquid

90   chromatography (FPLC) system (GE Healthcare) equilibrated with the standard buffer.

91   The supernatant was applied to a HiLoad 26/60 Superdex 200 prep-grade column (GE

92   Healthcare) that had already been equilibrated with standard buffer and eluted.

93   CLA-DH was further purified using a Mono Q 10/100 GL column (GE Healthcare), a

94   Superdex 200 10/300 GL column (GE Healthcare), and a Phenyl Superose HR 10/10

95   (Pharmacia). The purified CLA-DH was dialyzed with the standard buffer including

96   50% (v/v) glycerol and stored at $-20°C$ until further use.

97

98   **2.3. Determination of the molecular mass of CLA-DH**

99   In order to determine the native molecular mass of CLA-DH, the enzyme solution

100   was subjected to high performance gel-permeation chromatography on a G-3000SW

101   column (0.75×60 cm, Tosoh, Tokyo, Japan) at room temperature. It was eluted with

102   100 mM KPB (pH 6.5) containing 100 mM Na_2SO_4 at a flow rate of 0.5 mL/min. The

103   absorbance of the effluent was monitored at 280 nm. The molecular mass of the

104   enzymes was determined from their mobility relative to those of standard proteins.

105

106   **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

125 by measuring enzyme activity after incubating at 37°C for 10 min in the following

126 buffers under anaerobic conditions: sodium citrate buffer (50 mM; pH 3.0–4.0), sodium

127 succinate buffer (50 mM; pH 4.0–6.0), KPB (50 mM; pH 5.5–8.0), and Tris-HCl buffer

128 (50 mM; pH 7.0–9.0).

129

130 **2.5. Kinetic analysis**

131   All procedures were performed in an anaerobic chamber. Reactions were

132 performed under standard reaction conditions with modified substrate and enzyme

133 concentrations. The kinetics of HYA dehydrogenation were studied using 30–1000 µM

134 HYA complexed with 0.02% (w/v) BSA as the substrate, 7 µg/mL CLA-DH, and a

135 reaction time of 15 min. The kinetics of KetoA dehydrogenation were studied using

136 1–20 µM KetoA complexed with 0.02% (w/v) BSA as the substrate, 0.35 µg/mL

137 CLA-DH, and a reaction time of 5 min. The kinetic parameters were calculated by using

138 the experimental data with the Michaelis–Menten equation using KaleidaGraph 4.0

139 (Synergy Software Inc., PA, USA).

140

141 **2.6. Lipid analysis**

142   Before lipid extraction, *n*-heptadecanoic acid was added to the reaction mixture as an

143 internal standard. Lipids were extracted from 1 mL of the reaction mixture using 5 mL

144 of chloroform/methanol/1.5% (w/v) KCl in H_2O (2:2:1, by volume) according to the

145 procedure of Bligh-Dyer, and then concentrated by evaporation under reduced pressure

146 [16]. The resulting lipids were dissolved in 5 mL of benzene/methanol (3:2, by volume)

147 and methylated with 300 μL of 1% trimethylsilyldiazomethane (in hexane) at 28°C for

148 30 min. After methyl esterification, the resulting fatty acid methyl esters were

149 concentrated by evaporation under reduced pressure. The resulting fatty acid methyl

150 esters were analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto,

151 Japan) GC-1700 gas chromatograph equipped with a flame ionization detector, a split

152 injection system, and a capillary column (SPB-1, 30 m × 0.25 mm I.D., SUPELCO, PA,

153 USA). The initial column temperature 180°C (for 30 min) was subsequently increased

154 to 210°C at a rate of 60°C/min, and then maintained at 210°C for 29.5 min. The injector

155 and detector were operated at 250°C. Helium was used as a carrier gas at a flow rate of

156 1.4 mL/min. The fatty acid peaks were identified by comparing the retention times to

157 those of known standards.

158

159 **2.7. Enantiomeric purity analysis of hydroxy fatty acids**

160 The enantiomeric purity of HYA, which was produced from KetoA hydrogenation

161 with CLA-DH, was analyzed using HPLC (Shimadzu, Kyoto, Japan) using a Shimadzu

162 LC 20A System (Shimadzu) equipped with a chiral column (Chiralpak IA, 4.6 mm I.D.,

163 Daicel, Osaka, Japan) and an Evaporative Light Scattering Detector System (Shimadzu,

164 Kyoto, Japan) as a detector. Acetonitrile/0.2% formic acid (65:35) was used as a solvent

165 at a flow rate of 1.0 mL/min. Racemic HYA prepared from KetoA reduction with

166 NaBH$_4$ [17] was used as the standard.

167

168 **3. Results**

169 **3.1. Purification of CLA-DH**

170 The recombinant CLA-DH without the tag was purified to homogeneity from

171 cell-free extracts of the transformed *E. coli* through four steps of column

172 chromatography. The purified CLA-DH displayed a single band on an SDS-PAGE gel

173 (Fig. 1). The observed molecular mass of the subunit was 40 kDa, corresponding to a

174 calculated mass of 32 kDa deduced from the amino acid sequence of its gene. The

175 relative native molecular mass was estimated to be 32 kDa by HPLC on a G-3000SW

176 column, indicating that the enzyme consists of the single subunit. The purified CLA-DH

177 was used for further characterization.

178

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

11

197 HYA, (*R*)-HYA, 10-hydroxyoctadecanoid 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    $7.6 \cdot 10^{-3}$ 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 $5.7 \cdot 10^{-1}$ 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

233

234   We revealed polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU

235   1009a and identified CLA-DH. The CLA-DH gene was located together with CLA-DC

236   (fatty acid isomerase) and CLA-ER (fatty acid enone reductase) genes involved in

237   polyunsaturated fatty acid saturation metabolism in *L. plantarum* AKU 1009a [6]. These

238   results suggested that CLA-DH plays an important role in saturation metabolism.

239   CLA-DH, which belongs to the short-chain dehydrogenase/reductase (SDR) family,

240   showed considerable similarity with other SDRs (Fig. 4). In this paper, we characterized

241   CLA-DH from the aspect of its physiological function to clarify its distinct

242   characteristic properties in the SDR family, especially from the viewpoint of substrate

243   specificity. There are few reports regarding either hydroxy fatty acid dehydrogenation or

244   oxo fatty acid hydrogenation in the SDR family. However, CLA-DH catalyzed the

245   dehydrogenation or hydrogenation of fatty acids which have an internal hydroxy or an

246   oxo group, respectively (Table 1 and 2). *Micrococcus luteus* WIUJH-20 was reported to

247   convert 10- or 12-hydroxyoctadecanoic acid to the corresponding oxooctadecanoic acid.

248   The amino acid sequence of the enzyme which catalyzes the above oxidation of

249   hydroxy fatty acid in *M. luteus* WIUJH-20 belongs to a secondary alcohol

250   dehydrogenase [18]. The amino acid sequence of the secondary alcohol dehydrogenase

14

251 from *M. luteus* did not resemble that of CLA-DH, indicating that the dehydrogenation

252 activity of CLA-DH was characteristic activity among the SDR family.

253   As a characteristic property of CLA-DH, the enzyme showed higher activity in

254 hydrogenation than dehydrogenation reactions. The activity of KetoA hydrogenation

255 was 5 times higher than that of HYA dehydrogenation (Fig. 2).

256   Although many SDRs have high enantioselectivity [19-23], CLA-DH had low

257 enantioselectivity to dehydrogenate both (*R*) and (*S*) hydroxy fatty acids (Table1) and

258 produce (*R*) and (*S*) hydroxy fatty acids from oxo fatty acid. In addition, CLA-DH

259 dehydrogenated 10-, 12-, and 13-hydroxy fatty acids (Table 1), indicating its low

260 regioselectivity.

261   In our previous study, we reported the production of many kinds of hydroxy fatty

262 acids such as 10- and 13-hydroxy octadecapolyenoic acid [6,11,13,14]. Using these

263 various hydroxy fatty acids and hydroxy fatty acid dehydrogenase such as CLA-DH, we

264 can provide many kinds of corresponding oxo fatty acids by applying the wide substrate

265 specificity of CLA-DH. These results enable us to provide new functional lipids, oxo

266 fatty acids.

267

268 **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

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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

359 circles) and hydrogenation activity (open circles) were assessed under standard reaction

360 conditions after incubation at each temperature (18°C –67°C) for 30 min. The activities

361 after incubation at 18°C were defined as 100% for dehydrogenation (0.048 U/mg) and

362 hydrogenation (0.22 U/mg). (b) Effect of pH: The pH stabilities of the dehydrogenation

363 (closed) and hydrogenation (open) reactions were evaluated under standard reaction.

364 conditions after incubation at 37°C for 10 min at each pH. Sodium citrate buffer, pH

365 3.0–4.0 (circles), sodium succinate buffer, pH 4.0–6.0 (triangles), potassium phosphate

366 buffer, pH 5.0–7.5 (diamonds), and Tris-HCl buffer, 7.0–9.0 (squares) were used. The

367 activities after incubation in sodium succinate buffer (pH 4.5) were defined as 100% for

368 dehydrogenation (0.042 U/mg) and hydrogenation (0.22 U/mg).

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

370 **family**

371 The SDR family includes *Rhodococcus erythropolis* (AADH), *Lactobacillus brevis*

372 (LbRADH), *Thermus thermophilus* (TtADH), and *Leifsonia* sp. strain S749 (LSADH).

373 The accession numbers of the listed proteins are as follows: CLA-DH, BAL42247;

374 AADH, BAF43657; LbRADH, YP_794544; TtADH, YP_003977; LSADH, BAD99642.

375 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

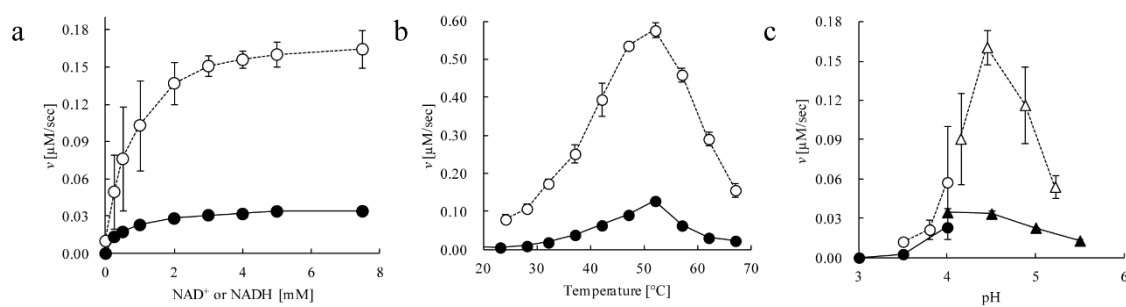377   members of catalytic tetrad.

378

Marker

97,400

66,300

42,400

← CLA-DH

30,000

20,100

379
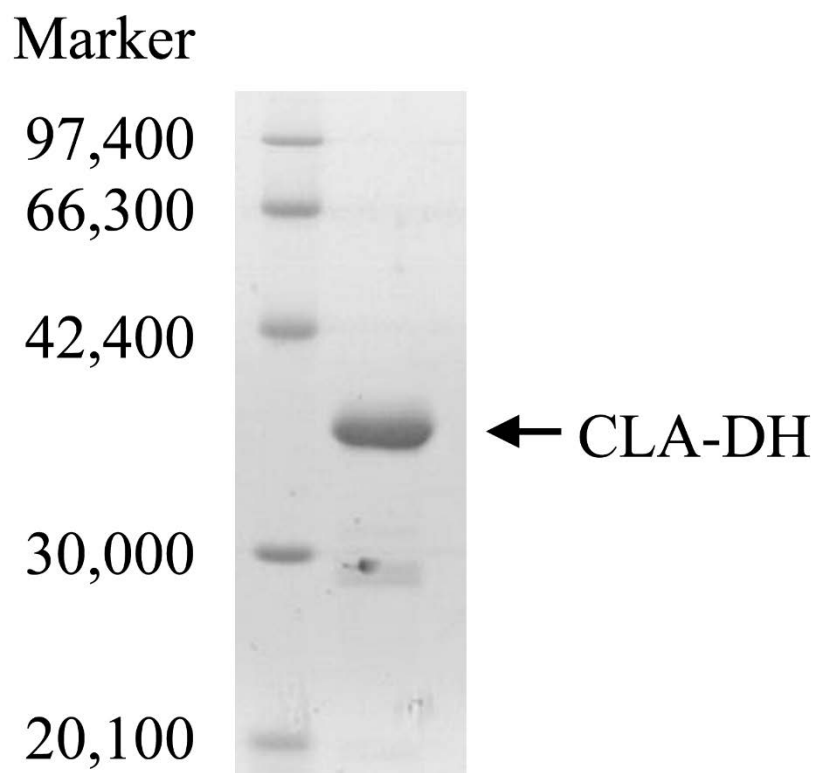
Fig. 1 SDS-PAGE analysis of purified CLA-DH

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Fig. 2 Effects of NAD$^+$/NADH concentrations, temperature, and pH on the activity
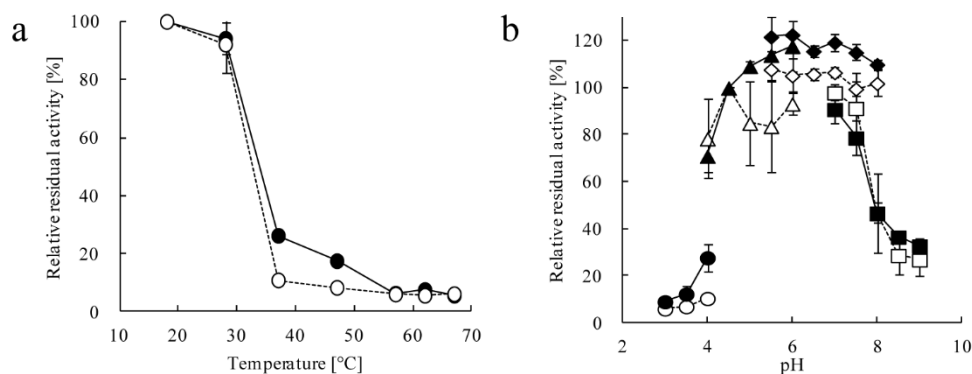
384

of CLA-DH

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

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CLA-DH    1 --MKDFKDKVMFITGAAHGFGQVIAEGAADRGMKLTIVDIDEPALKKTYQHILDKGAEVL  58
AADH      1 -MFNSIEGRSVVVTGGSKGIGLGMVRVFARAGANVLMTARDALTLERAAEGLNGLPGAVS  59
LbRADH    1 -MSNRLDGKVAIVTGGTLGIGLAIATKFVEEGAKVMITGRHSDVGEKAAKSVGTPDQIQF  59
TtADH     1 MGLF--AGKGVLVTGGARGIGRAIAQAFAREGALVALCDLRPEGKE--VAEAIGGAFFQV  56
LSADH     1 MAQYDVADRSAIVTGGGSGIGRAVALTLAASGAAVLVTDLNEEHAQAVVAEIEAAGGKAA  60
                          TGXXXGXG
CLA-DH   59 MVTADVTKEASVDDAVEQAMEKFGRIDLLINNAGIALP-GRIWELPTRDWEWIMHINLMS 117
AADH     60 TLQVDVTNPDSLAGMAEVAAERHGGIDVLCANAGIFPS-KRLGEMTSEDMDSVFGVNVKG 118
LbRADH   60 FQHDSSDED-GWTKLFDATEKAFGPVSTLVNNAGIAVN-KSVEETTTAEWRKLLAVNLDG 117
TtADH    57 DLEDERERV-RFVEE---AAYALGRVDVLVNNAAIAAPGSAL-TVRLPEWRRVLEVNLTA 111
LSADH    61 ALAGDVTDP-AFGEASVAGANALAPLKIAVNNAGIGGEAATVGDYSLDSWRTVIEVNLNA 119
                                     *
CLA-DH  118 QVYAMKRVIPIMIQQKTHADILNVASIAGLVDT-PGMPSYHASKFASVGMTEATAYDLQR 176
AADH    119 TIHAVQACMPWLETSGRGRVVVTS-SITGPVTGYPGWSHYGASKAAQMGFIRTAAIELAP 177
LbRADH  118 VFFGTRLGIQRMKNKGLGASIINMSSIEGFV-GDPSLGAYNASKGAVRIMSKSAALDCAL 176
TtADH   112 PMHLSALAAREMRKVG-GGAIVNVASVQGLF-AEQENAAYNASKGGLVNLTRSLALDLAP 169
LSADH   120 VFYGMQPQLKAMAANG-GGAIVNMASILGSV-GFANSSAYVTAKHALLGLTQNAALEYAA 177
                                   *               *      *
CLA-DH  177 ANIDIDMHVMCPGFVQTDLYHTENHRPAQYSDPTDPYYQSEAYLKGQQFAKYVITNGKPI 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 DIANAALFFALDEAAYITG-------------QSLIVDGGQVLPESAMALGEL     259
LbRADH  222 DIAYICVYLASNESKFATG-------------SEFVVDGGYTAQ---------     252
TtADH   218 EVAEAVLFLASEKASFITG-----------AILPVDGGMTASFMMAGRPV-     256
LSADH   221 EVASLVAFLASDAASFITG-----------SYHLVDGGYTAQ---------     251
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Fig. 4 Multiple-sequence alignment of CLA-DH and ADHs belonging to the SDR family

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395 Table 1 **Substrate specificity of CLA-DH for dehydrogenation.**

Substrate	Relative activity [%]
397 (S)-10-Hydroxy-cis-12-octadecenoic acid (HYA)	100 [a]
398 (R)-10-Hydroxy-cis-12-octadecenoic acid ((R)-HYA)	98
399 10-Hydroxyoctadecanoic acid	25
400 10-Hydroxy-trans-11-octadecenoic acid	69
401 (S)-10-Hydroxy-cis-12,cis-15-octadecadienoic acid	75
402 (S)-10-Hydroxy-cis-6,cis-12-octadecadienoic acid	54
403 (R)-12-Hydroxy-cis-9-octadecenoic acid	62
404 13-Hydroxy-cis-9-octadecenoic acid	142
405 3-Hydroxyoctadecanoic acid (C18)	- [b]
406 3-Hydroxytetradecanoic acid (C14)	-
407 2-Hydroxyeicosanoic acid (C20)	-
408 Methyl (S)-10-Hydroxy-cis-12-octadecenoate	127
409 8-Hexadecanol	24

410 [a], The activity of (S)-10-hydroxy-cis-12-octadecenoic acid dehydrogenation (=0.048

411 U/mg) under the condition (5 mM NAD^+; 37°C, pH 4.5, 15 min) was defined as 100%.

412 [b]-, not detected.

413

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

Substrate	Relative activity [%]
415 416 10-Oxo-*cis*-12-octadecenoic acid (KetoA)	100 [a]
417 10-Oxooctadecanoic acid	66
418 10-Oxo-*trans*-11-octadecenoic acid	53
419 10-Oxo-*cis*-12,*cis*-15-octadecadienoic acid	44
420 10-Oxo-*cis*-6,*cis*-12-octadecadienoic acid	6
421 12-Oxo-*cis*-9-octadecenoic acid	87
422 13-Oxo-*cis*-9-octadecenoic acid	156
423 Methyl 10-oxo-*cis*-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%.

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