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Title	Characterization of the linoleic acid Δ9 hydratase catalyzing the first step of polyunsaturated fatty acid saturation metabolism in Lactobacillus plantarum AKU 1009a.
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1   Characterization of the linoleic acid Δ9 hydratase catalyzing the first step of

2   polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU

3   1009a

4   Running title: Linoleic acid Δ9 hydratase from *L. plantarum*

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18   Dehydration

19

ABSTRACT

Linoleic acid Δ9 hydratase, which is involved in linoleic acid saturation metabolism of *Lactobacillus plantarum* AKU 1009a, was cloned, expressed as a his-tagged recombinant enzyme, purified with an affinity column, and characterized. The enzyme required FAD as a co-factor and its activity was enhanced by NADH. The maximal activities for the hydration of linoleic acid and for the dehydration of 10-hydroxy-*cis*-12-octadecenoic acid (HYA) were observed at 37°C in buffer at pH 5.5 containing 0.5 M NaCl. Free C16 and C18 fatty acids with *cis*-9 double bonds and 10-hydroxy fatty acids served as substrates for the hydration and dehydration reactions, respectively. The apparent K_m value for linoleic acid was estimated to be 92 μM, with a k_{cat} of $2.6 \cdot 10^{-2}$ sec^{-1} and a Hill factor of 3.3. The apparent K_m value for HYA was estimated to be 98 μM, with a k_{cat} of $1.2 \cdot 10^{-3}$ sec^{-1}.

INTRODUCTION

Hydroxy fatty acids are derived from a variety of natural sources, including microorganisms, plants, animals, and insects; they are found in triacylglycerols, waxes, cerebrosides, and other lipids. Hydroxy fatty acids are versatile starting materials that have been utilized to produce resins, waxes, nylons, plastics, lubricants, biopolymers, and biodiesel (1). They also have useful antibiotic, anti-inflammatory, and anticancer activities (2). For example, ricinoleic acid (12-hydroxy-*cis*-9-octadecenoic acid), derived from castor oil, is converted to sebacic acid (decanedioic acid), the monomer for nylon synthesis, and has anti-inflammatory and antinociceptive activity (3).

Many microorganisms can convert oleic acid (*cis*-9-octadecenoic acid) into 10-hydroxyoctadecanoic acid (1). It has been recently reported that the

2

44 myosin-crossreactive antigens (MCRAs) from *Elizabethkingia meningoseptica* (4),

45 *Streptococcus pyogenes* (5), and *Bifidobacterium breve* (6) can also convert oleic acid

46 into 10-hydroxyoctadecanoic acid. The hydration of unsaturated fatty acids has been

47 suggested to be a detoxification mechanism in bacteria harboring MCRA proteins and a

48 survival strategy for living in fatty acid-rich environments (5), indicating that MCRA

49 proteins are fatty acid hydratases. A few of the microorganisms having an MCRA

50 protein, including *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Streptococcus*

51 *bovis*, *Nocradia cholesterolicum*, *Pediococcus pentosaceus*, and *Pediococcus* sp. can

52 convert   linoleic   acid   (LA,   *cis*-9,*cis*-12-octadecadienoic   acid)   to

53 10-hydroxy-*cis*-12-octadecenoic acid (HYA), 13-hydroxy-*cis*-9-octadecenoic acid, or

54 10,13-dihydroxyoctadecanoic acid (7–12).

55 　　We found that HYA is the initial intermediate in the biosynthesis of conjugated

56 linoleic acid (CLA) from LA in *L. acidophilus* (8). CLAs, which are isomers of LA,

57 have beneficial effects, such as preventing tumorigenesis (13) and arteriosclerosis (14)

58 and decreasing body fat content (15). In our previous study, we screened lactic acid

59 bacteria for the ability to produce CLA from LA, and selected *Lactobacillus plantarum*

60 AKU 1009a as a potential strain (16–18). *L. plantarum* AKU 1009a can transform not

61 only LA but also α-linolenic acid (*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid) and

62 γ-linolenic   acid   (*cis*-6,*cis*-9,*cis*-12-octadecatrienoic   acid)   into   the   corresponding

63 conjugated fatty acids (19–24). We also showed that CLA is synthesized, in part,

64 through the reactions of a newly discovered polyunsaturated fatty acid saturation

65 metabolism in *L. plantarum* AKU1009a (25, 26). The novel saturation metabolism

66 consisted   of   four   enzymes: CLA-HY   (hydratase/dehydratase),   CLA-DH

67 (dehydrogenase), CLA-DC (isomerase), and CLA-ER (enonereductase) (25, 26).

68 CLA-HY, CLA-DH, and CLA-DC are responsible for CLA synthesis from LA (25) and

69 CLA-ER is a key enzyme for the saturation metabolism (26).

70   In this study, we describe the enzymatic and physiochemical characteristics of

71 CLA-HY, which catalyzes the initial step of the saturation metabolism: the hydration of

72 LA and the dehydration of HYA. The enzyme was found to be a unique

73 hydratase/dehydratase demonstrating activity in the presence of FAD and NADH.

74

75 **MATERIALS AND METHODS**

76

77 **Chemicals**

78   HYA was prepared as previously described (8, 17). LA and fatty acid-free

79 (<0.02%) bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA).

80 All of the other chemicals were analytical grade and obtained commercially.

81

82 **Cloning and expression of recombinant proteins in *E. coli***

83   Primers were designed to amplify the CLA-HY sequence, without a stop codon,

84 from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product was ligated

85 into expression vector pET101/D-TOPO (Invitrogen, CA, USA), according to the

86 manufacturer's instruction. The integrity of the cloned gene was verified by DNA

87 sequencing using a Beckman-Coulter CEQ8000 (Beckman-Coulter, Fullerton, CA,

88 USA). The resulting plasmid was purified, and then used to transform *E. coli*

89 Rosetta^{TM}2 (DE3) (Novagen, WI, USA). The transformed cells were cultured in

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

91 isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM.

92   After adding IPTG, the transformed cells were cultivated at 20°C for 8 h with shaking at

93   100 rpm.

94

95   **Preparation of CLA-HY**

96   All purification procedures were performed at 4°C. The transformed cells (8 g) from

97   1.5 L of culture broth were harvested, suspended in binding buffer (16 mL), and treated

98   4 times, for 5 min each, with an ultrasonic oscillator (Insinator 201 M; Kubota, Japan).

99   The binding buffer contained 50 mM imidazole in 20 mM potassium phosphate buffer

100   (KPB) (pH 7.4). The cell debris was removed by centrifugation at 1700 $\times g$ for 10 min.

101   The resulting supernatants were used as cell-free extracts. The cell-free extracts were

102   fractioned by ultracentrifugation at 100,000 $\times g$ for 60 min and the supernatant was

103   obtained. The enzyme was purified from the supernatant using a fast protein liquid

104   chromatography (FPLC) system (Amercham Pharmacia Biotech Co., Uppsala, Sweden)

105   equipped with a His Trap HP column (GE Healthcare, Buckinghamshire, England). The

106   column was equilibrated with the binding buffer and the fractions containing CLA-HY

107   were eluted with elution buffer containing 250 mM imidazole in 20 mM KPB (pH 7.4).

108   The fractions containing CLA-HY were collected and dialyzed against 20 mM KPB (pH

109   6.5).

110

111   **Determination of the molecular mass of His-tagged CLA-HY**

112   In order to determine the native molecular mass of His-tagged CLA-HY, the enzyme

113   solution was subjected to high performance gel-permeation chromatography on a

114   G-3000SW column (0.75 $\times$ 60 cm, Tosoh, Tokyo, Japan) at room temperature. It was

115   eluted with 100 mM KPB (pH 6.5) containing 100 mM Na_2SO_4 at the flow rate of 0.5

116   mL/min. The absorbance of the effluent was monitored at 280 nm. The molecular mass

117   of the enzymes was determined from its mobility relative to those of standard proteins.

118

119   **Reaction conditions**

120   All operations were performed in an anaerobic chamber. The standard reaction

121   conditions were as follows. The reactions were performed in test tubes (16.5 × 125 mm)

122   that contained 1 mL of reaction mixture (20 mM sodium succinate buffer, pH 5.5) with

123   0.5% (w/v) LA or 0.1% (w/v) HYA complexed with BSA [0.1% (w/v) or 0.02% (w/v),

124   respectively] as the substrate, 5 mM NADH, 0.1 mM FAD, and 40 µg (= 0.04 U)/mL

125   CLA-HY. One unit was defined as the amount of enzyme that catalyzes the conversion

126   of 1 µmol of LA per min. The reactions were performed under anaerobic conditions in a

127   sealed chamber containing an O_2-absorbent (Aneropack "Kenki," Mitsubishi Gas

128   Chemical Co., Ltd., Tokyo, Japan). The reaction mixture was gently shaken (120

129   strokes/min) at 37°C for 30 min (for hydration) or 15 min (for dehydration). All

130   experiments were performed in triplicates, and the averages of three separate

131   experiments that were reproducible within ±10% are presented in the figures and tables.

132

133   **Enzyme activity assay**

134   Reactions were performed under the standard reaction conditions with some

135   modifications, as described below. The effects of cofactors were examined using a

136   reaction mixture containing LA or HYA as the substrate and various cofactors, such as

137   0.1 mM FMN, 0.1 mM FAD, 5 mM NADH, 5 mM NADPH, 5 mM NAD^+, and 5 mM

138   $NADP^+$, in various combinations. The effect of oxygen was examined by comparing the

139   reactions under anaerobic condition and aerobic condition. The optimal reaction

140   temperature was examined by incubating the reaction mixture (20 mM sodium succinate

141   buffer, pH 5.5) at various temperatures for 30 min (for hydration) or 15 min (for

142   dehydration) under anaerobic conditions. The optimal reaction pH was determined at

143   37°C using 20 mM sodium succinate buffer (pH 4.5–6.0) and 20 mM KPB (pH 5.5–7.0).

144   The effect of NaCl concentration was examined by measuring the enzyme activity of

145   reaction mixtures containing NaCl (0–1 M). Thermal stability was determined by

146   measuring the enzyme activity after incubating reaction mixtures containing 20 mM

147   sodium succinate buffer (pH 5.5) and 0.1 mM FAD at various temperatures for 30 min

148   under anaerobic condition. The pH stability was determined by measuring the enzyme

149   activity after incubating at 37°C for 10 min in the following buffers under anaerobic

150   conditions: sodium citrate buffer (50 mM; pH 3.0–4.0), sodium succinate buffer (50

151   mM; pH 4.0–6.0), KPB (50 mM; pH 5.0–8.0), and Tris-HCl buffer (50 mM; pH

152   7.0–9.0).

153

154   **Kinetic analysis**

155   All operations were performed in an anaerobic chamber. Reactions were

156   performed under standard reaction conditions with modified substrate concentrations.

157   The kinetics of LA hydration were studied using 50–400 µM LA complexed with 0.1%

158   (w/v) BSA as the substrate and reaction times of 15 min. The kinetics of HYA

159   dehydration were studied using 50–400 µM HYA complexed with or 0.02% (w/v) BSA

160   as the substrate and reaction times of 30 min. The kinetic parameters were calculated by

161   fitting the experimental data to the Hill equation or the Michaelis-Menten equation

162   using KaleidaGraph 4.0 (Synergy Software Inc., PA, USA).

163

164 **Lipid analysis**

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

166 an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL

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

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

169 (27). The resulting lipids were dissolved in 1 mL of dichloromethane and methylated

170 with 2 mL of 4% methanolic HCl at 50°C for 20 min. After adding 1 mL of water, the

171 resulting fatty acid methyl esters were extracted with 5 mL of *n*-hexane and

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

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

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

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

176 USA). The initial column temperature, 180°C for 30 min, was subsequently increased to

177 210°C at a rate of 60°C/min, and then maintained at 210°C for 29.5 min. The injector

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

179 1.4 mL/min. The fatty acid peaks were identified by comparing the retention times to

180 those of known standards.

181

182 **Enantiomeric purity analysis of hydroxy fatty acids**

183   The enantiomeric purities of HYA, 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid,

184 and 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid were analyzed by Mosher's method

185 (28) using a Bruker Avance III 500 (500MHz) NMR.

186

187 **RESULTS**

Purification of CLA-HY

188

189   The relative molecular mass was calculated to be 52 kDa by high performance

190   gel-permeation chromatography on a G-3000SW column. Purified CLA-HY displayed a

191   single band on an SDS-PAGE gel (Fig. 1). Observed molecular weight of purified

192   CLA-HY was 66 kDa, which was corresponding to calculated mass of 68 kDa deduced

193   from the amino acid sequence of its gene. The purified CLA-HY was used for

194   enzymatic characterization.

195

Effects of cofactors and oxygen

196

197   The effects of potential cofactors FMN, FAD, NADH, NADPH, NAD^+, and $NADP^+$

198   were examined (Fig. 2). Hydration and dehydration activity were observed only in the

199   reaction mixtures containing FAD. The addition of NADH or NADPH with FAD

200   increased these activities by a factor of approximately thirty. The effect of oxygen was

201   examined using reaction mixtures containing FAD and NADH under aerobic conditions.

202   The activity under aerobic conditions was 40–50% of that under anaerobic conditions,

203   which were maintained in a sealed chamber with an O_2 absorbent (Fig. 3).

204

Effects of reaction conditions

205

206   The effects of pH on the activity of CLA-HY were examined over the pH range

207   from 4.5 to 7.0 (Fig. 4a). The enzyme showed maximal activity at pH 5.5. The effects of

208   reaction temperature on the activity of CLA-HY were also examined. The optimal

209   temperature was found to be 37–42°C (Fig. 4b). The effects of FAD concentration were

210   examined from 0 to 100 µM FAD, with or without NADH (Fig. 4c). The hydration and

211   dehydration activities were 10 times greater with NADH than that without NADH. The

9

212   enzyme activity increased with increasing FAD concentration up to 20 µM with NADH

213   and up to 1 µM without NADH. The activities remained the same at higher

214   concentrations of FAD, except in the case of hydration without NADH, which showed

215   decreasing activity with concentrations of FAD above 1 µM. The effect of NADH

216   concentration was examined from 0 to 5 mM (Fig. 4d). The hydration and dehydration

217   activities increased with increasing NADH concentration. The effects of NaCl

218   concentration was examined from 0 to 1M (Fig. 4e). The enzyme showed its highest

219   activity with 0.5 M NaCl, which was three times higher than that without NaCl.

220

221   **Enzyme stability**

222   The thermal stability of the purified enzyme was investigated from 4°C to 42°C. The

223   enzyme was incubated at each temperature with or without FAD (Fig. 5a). The enzyme

224   was more stable with FAD than that without FAD. More than 80% of the initial activity

225   remained at temperatures up to 32°C in the presence of FAD, and at temperatures up to

226   28°C in the absence of FAD. The pH stability of the purified enzyme was investigated

227   by incubating the enzyme in different buffers within the pH range 3.0 to 9.0. More than

228   80% of the initial activity remained in the pH range 4.5 to 6.5 (Fig. 5b).

229

230   **Substrate specificity**

231   In the hydration reaction, free C16 and C18 fatty acids with a *cis* carbon–carbon

232   double bond at the Δ9 position, such as LA, palmitoleic acid (*cis*-9-hexadecenoic acid),

233   oleic acid, α-linolenic acid, γ-linolenic acid, stearidonic acid, and ricinoleic acid, served

234   as substrates and were transformed into the corresponding 10-hydroxy fatty acids. In

235   contrast, fatty acids with a *trans* carbon–carbon double bond at Δ9 position (elaidic acid,

236 *trans*-9-octadecenoic acid), fatty acid esters (methyl linoleate, monolinolein, dilinolein,

237 and trilinolein), and conjugated fatty acids (conjugated linoleic acids) were not hydrated.

238 Fatty acids with other chain lengths, such as myristoleic acid (*cis*-9-tetradecenoic acid),

239 arachidonic   acid   (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic   acid),   EPA

240 (*cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosapentaenoic   acid),   and   DHA

241 (*cis*-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-docosahexaenoic acid) were not hydrated; nor

242 were fatty acids with a *cis* carbon-carbon double bond at $\Delta 11$ position, such as

243 *cis*-vaccenic acid and *cis*-11-octadecenoic acid, or fatty alcohols, such as linoleyl

244 alcohol (Table 1).

245   In the dehydration reaction, 10-hydoxy C18 fatty acids, such as HYA,

246 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic   acid,

247 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid, and 10-hydroxyoctadecanoic acid served

248 as substrates and were transformed into the corresponding fatty acids with *cis* double

249 bonds at the $\Delta 9$ position. However, 12-hydroxy, 3-hydroxy, and 9-hydroxy fatty acids

250 were not dehydrated (Table 2).

251

252 **Kinetic analysis of the CLA-HY catalyzing reactions**

253   The substrate–velocity curve for LA hydration had a sigmoid shape. When fitted

254 with the Hill equation, the apparent K_m value for LA was estimated to be 92 μM with a

255 k_{cat} value of $2.6 \cdot 10^{-2}$ sec^{-1} and a Hill factor of 3.3. The apparent K_m value for HYA in the

256 dehydration reaction was estimated to be 98 μM with a k_{cat} of $1.2 \cdot 10^{-3}$ sec^{-1}.

257

258 **Enantiomeric purities of the produced hydroxy fatty acids**

259   The carbons bearing hydroxy functional groups in the hydroxy fatty acids

11

260 produced during the hydration reaction are asymmetric. The enantiomeric purities of the

261 HYA,   10-hydroxy-*cis*-12,*cis*-15-octadecadienoic   acid,   and

262 10-hydroxy-*cis*-6,*cis*-12-octadecadienoic acid produced by CLA-HY were analyzed

263 using Mosher's method. They were found to be of the (*S*)-configuration with more than

264 99.9% enantiomeric excess (*e.e.*).

265

266 **Effects of chemicals on the enzyme activity**

267   The effects of monovalent and divalent metal ions (1 mM) were investigated in both

268 the hydration and dehydration reactions. The reactions were strongly inhibited by Ag^+,

269 Fe^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, and Fe^{3+} (data not shown). Dodecanoic acid was reported to

270 form an insoluble complex with metal ions (Cu^{2+}, Zn^{2+}, Fe^{3+}, Co^{2+}, and Ni^{2+}) (29). One

271 of the reasons these metal ions inhibited the reaction may have been the formation of

272 substrate complexes like those observed with dodecanoic acid. In contrast, a slight

273 increase in the hydration activity was observed with $MnCl_2$ (1 mM).

274   The effects of various enzyme inhibitors (1 mM), such as SH-reagents, carbonyl

275 reagents, serine protease inhibitors, and redox indicators, were investigated. Significant

276 inhibition was found only with triphenyl tetrazolium chloride, a redox indicator that can

277 serve as a strong electron acceptor.

278

279 **DISCUSSION**

280   Many bacteria have an MCRA protein to detoxify unsaturated fatty acids by

281 transforming them into hydroxy fatty acids. CLA-HY, which belongs to the MCRA

282 family, should also detoxify unsaturated fatty acids in *L. plantarum*, because the growth

283 of *L. plantarum* is inhibited by LA (16).

284   CLA-HY required FAD for activity; its activity was further increased by the

285   addition of NADH (Fig. 2 and Fig. 4c). These results indicate that CLA-HY is an

286   FAD-dependent enzyme, and NADH is an activator of CLA-HY. Almost all MCRA

287   proteins have an FAD-binding motif, such as a GXGXXS(A/G) (30), but the

288   corresponding sequence in CLA-HY is GAGLSN. FAD might bind loosely to CLA-HY,

289   because the purified CLA-HY, after dialysis, showed no absorbance at 450 nm (data not

290   shown).

291   The absorbance of FAD at 450 nm was decreased by the addition of NADH,

292   indicating that $FADH_2$ is the active cofactor and is produced through the reduction of

293   FAD by NADH. Oxygen inactivated the enzyme because $FADH_2$ is easily oxidized by

294   oxygen (Fig. 3). The mechanism of NADH activation was similar to that described for

295   2-haloacrylate hydration by 2-haloacrylate hydratase (31). $FADH_2$ may be involved in

296   the activation of a water molecule that attacks the Δ9 double bond of LA, or in the

297   protonation of the C10 carbon of LA. As for dehydration, the hydroxy group of the

298   hydroxy fatty acid may be activated by $FADH_2$. In addition to this activation, $FADH_2$

299   may also have a role in stabilizing the enzyme through its reducibility.

300   There are few reports concerning the dehydration of hydroxy fatty acids. In many

301   cases, the substrates are 2-hydroxy or 3-hydroxy fatty acyl-CoAs, which are

302   intermediates in the elongation of fatty acids and are dehydrated by enoyl-CoA

303   dehydratase (32). In this paper, we described the enzymatic dehydration of 10-hydroxy

304   fatty acids for the first time. A detailed analysis of the CLA-HY-catalyzed reaction

305   provided novel information about enzymatic dehydration: the requirement for FAD and

306   activation by NADH. Such information may be useful for creating industrially

307   important dehydration catalysts for the synthesis of monomers for radical

308   polymerization. The possibilities include the synthesis of acrylamide, propylene, and

309   styrene from alcohols 3-hydroxypropionate/lactate, propanol, and ethanol, respectively.

310   The newly generated double bonds in the products of CLA-HY-catalyzed

311   dehydration were in the *cis*-configuration in this study, whereas the generation of double

312   bonds in the *trans*-configuration was observed in our previous study (26). These results

313   indicate the possibility that some reaction conditions or additional factors affect the

314   geometric selectivity of the dehydration reaction. Because *trans* fatty acids are reported

315   to be harmful for health, the geometric selectivity of the hydration reaction must be

316   controlled to reduce *trans* fatty acids in dietary foods. We are investigating the factors

317   that control geometric selectivity in CLA-HY-catalyzed hydration. The results of the

318   detailed analysis will be presented in future reports.

319   Hydroxy fatty acids are important materials for the chemical, food, cosmetic,

320   and pharmaceutical industries; they have also attracted recent interest from a variety of

321   research fields. For example, hydroxy fatty acids can be applied to the production of

322   biopolymers, the improvement of health, and the production of pharmaceuticals with

323   anti-inflammatory   and   antinociceptive   effects.   Not   only   HYA   and

324   10-hydroxyoctadecanoic acid but also 10-hydroxy-*cis*-12,*cis*-15-octadecadienoic acid,

325   10-hydroxy-*cis*-6,*cis*-12-octadecadienoic   acid,

326   10-hydroxy-*cis*-6,*cis*-12,*cis*-15-octadecatrienoic acid, and 10,12-dihydroxyoctadecanoic

327   acid   can   be   produced   by   CLA-HY-catalyzed   reactions.   CLA-HY   showed

328   regioselectivity for Δ9 double bond hydration, generating C10 hydroxy groups in the

329   (*S*)-configuration with high enantioselectivity, while chemical hydration has some

330   difficulties with regio- and stereo-selectivities. These characteristics of CLA-HY enable

331   the fine synthesis of hydroxy fatty acids. These products with fine structures could be

14

useful for their precise functional evaluation for application purposes.

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448

449   **Figure legends**

450   Fig. 1 **SDS-PAGE analysis of purified CLA-HY.** Molecular mass standards: from the

451   top, phosphorylase b (97,200), bovine serum albumin (66,400), ovalbumin (45,000),

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452 carbonic anhydrase (29,000), and trypsin inhibitor (20,100). Observed molecular weight

453 of purified CLA-HY was 66 kDa.

454 Fig. 2 **Effects of cofactors on the activity of CLA-HY.** Hydration activity (black bars)

455 and dehydration activity (white bars) were assayed under standard reaction conditions

456 except for the addition of cofactors FMN, FAD, NADH, NADPH, NAD^+, $NADP^+$ in

457 various combinations.

458 Fig. 3 **Effect of oxygen on the activity of CLA-HY.** Hydration activity (black bars)

459 and dehydration activity (white bars) were assayed under standard reaction conditions.

460 Aerobic reactions were conducted in an open chamber. Anaerobic reactions were

461 performed in a sealed chamber with O_2-absorbent.

462 Fig. 4 **Effects of pH, temperature, and the concentration of FAD, NADH, and NaCl**

463 **on the activity of CLA-HY.** a, Effects of pH. Activity was assayed under standard

464 reaction conditions, except for the buffers used. Sodium succinate buffer (closed and

465 open circles for hydration and dehydration, respectively), pH 4.5–6.0, and potassium

466 phosphate buffer (closed and open triangles for hydration and dehydration, respectively),

467 pH 5.5–7.0, were used. b, Effects of temperature. Hydration activity (closed circles) and

468 dehydration activity (open circles) were assayed under standard reaction conditions,

469 except for the temperature. c, Effects of FAD concentration. Enzyme activity was

470 assayed under standard reaction conditions, except for the FAD concentration with

471 (closed and open circles for hydration and dehydration, respectively) or without (closed

472 and open triangles for hydration and dehydration, respectively) the addition of NADH.

473 d, Effects of NADH concentration. Hydration activity (closed circles) and dehydration

474 activity (open circles) were assayed under standard reaction conditions, except for the

475 NADH concentration. e, Effects of NaCl concentration. Hydration activity (closed

476 circles) and dehydration activity (open circles) were assayed under standard reaction

477 conditions, except for the addition of NaCl (0–1.0 M).

478 Fig. 5 **Effects of temperature and pH on stability of CLA-HY.** a, Effects of

479 temperature. The thermal stability of the hydration activity was assessed under standard

480 reaction conditions after incubation at each temperature (4–42°C) for 30 min with

481 (closed circles) or without (closed triangles) FAD. The thermal stability of the

482 dehydration activity was assessed under standard condition after incubation at each

483 temperature (4–42°C) for 30 min with (open circles) or without (open triangles) FAD.

484 The activities after incubation with FAD at 4°C or at 18°C were defined as 100% for

485 hydration (1.1 U/mg) and dehydration (0.020 U/mg), respectively. b, Effect of pH. The

486 pH stabilities of the hydration (closed) and dehydration (open) reactions were evaluated

487 under standard reaction conditions after incubation at 37°C for 10 min at each pH.

488 Sodium citrate buffer, pH 3.0–4.0 (circles), sodium succinate buffer, pH 4.0–6.0

489 (triangles), potassium phosphate buffer, pH 5.0–8.0 (diamonds), and Tris-HCl buffer,

490 7.0–9.0 (squares) were used. The activities after incubation in sodium succinate buffer

491 (pH5.5) and in sodium succinate buffer (pH 6.0) were defined as 100% for the hydration

492 (1.2 U/mg) and dehydration (0.13 U/mg), respectively.

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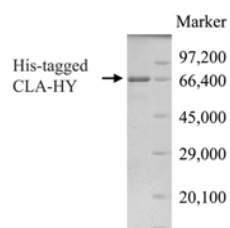Fig. 1 **SDS-PAGE analysis of purified CLA-HY.**
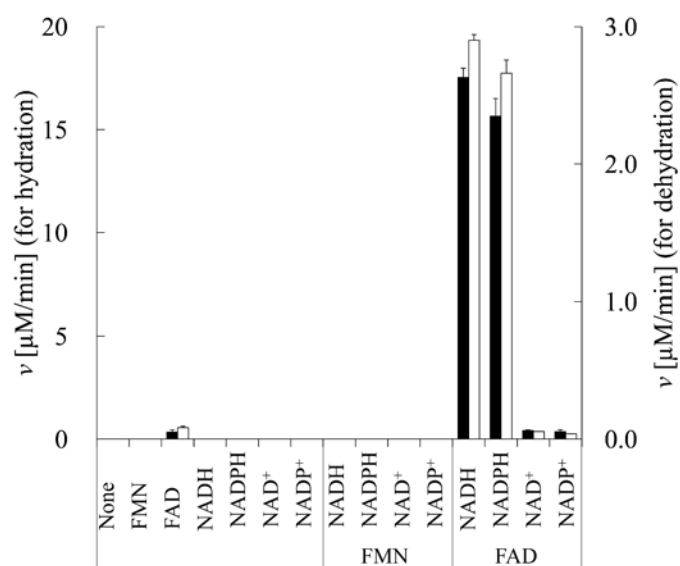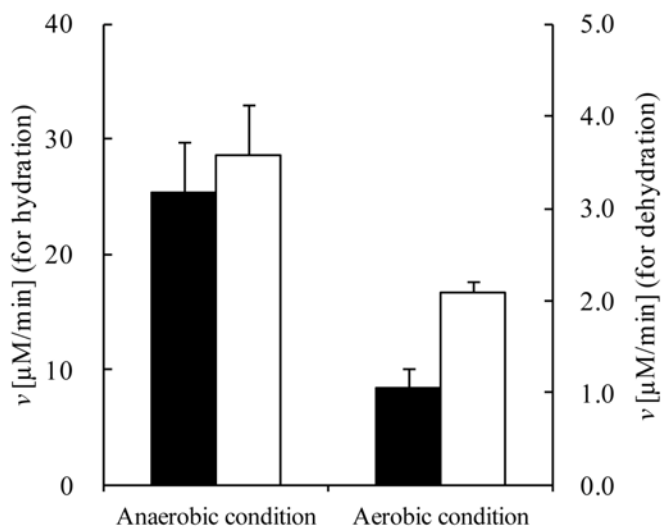
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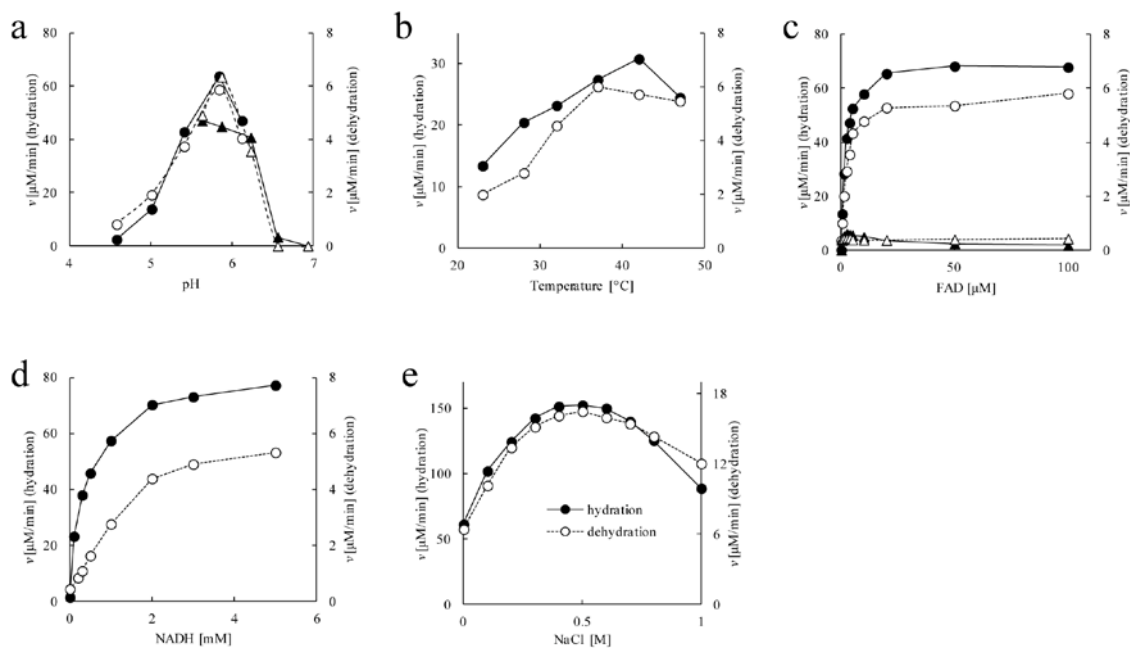Fig. 2 **Effects of cofactors on the activity of CLA-HY.**

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Fig. 3 **Effect of oxygen on the activity of CLA-HY.**

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Fig. 4 **Effects of pH, temperature, and the concentration of FAD, NADH, and NaCl**

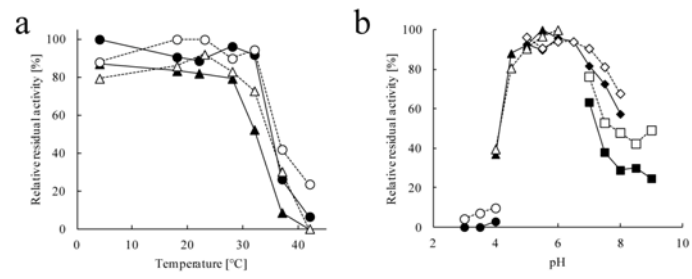**on the activity of CLA-HY.**

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513   Fig. 5 **Effects of temperature and pH on stability of CLA-HY.**

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515   Table 1 **Substrate specificity of CLA-HY-catalyzed hydration**

Substrate		Product	Relative activity [%]
cis-9,*cis*-12-Octadecadienoic acid	(Linoleic acid)	10-Hydroxy-*cis*-12-octadecenoic acid	100 [a]
cis-9-Tetradecenoic acid (C14)	(Myristoleic acid)		- [b]
cis-9-Hexadecenoic acid (C16)	(Palmitoleic acid)	10-Hydroxyhexadecanoic acid	44
cis-9-Octadecenoic acid (C18)	(Oleic acid)	10-Hydroxyoctadecanoic acid	335
trans-9-Octadecenoic acid	(Elaidic acid)		-
cis-9,*cis*-12,*cis*-15-Octadecatrienoic acid	(α-Linolenic acid)	10-Hydroxy-*cis*-12,*cis*-15-octadecadienoic acid	29
cis-6,*cis*-9,*cis*-12-Octadecatrienoic acid	(γ-Linolenic acid)	10-Hydroxy-*cis*-6,*cis*-12-octadecadienoic acid	43
cis-6,*cis*-9,*cis*-12,*cis*-15-Octadecatetraenoic acid	(Stearidonic acid)	10-Hydroxy-*cis*-6,*cis*-12,*cis*-15-octadecatrienoic acid	43
cis-5,*cis*-8,*cis*-11,*cis*-14-Eicosatetraenoic acid	(Arachidonic acid)		-
cis-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-Eicosapentaenoic acid	(EPA)		-
cis-4,*cis*-7,*cis*-10,*cis*-13,*cis*-16,*cis*-19-Docosahexaenoic acid	(DHA)		-
cis-9,*cis*-12-Octadecadienol	(Linoleyl alcohol)		-
12-Hydroxy-*cis*-9-octadecenoic acid	(Ricinoleic acid)	10,12-Dihydroxyoctadecanoic acid	0.5
cis-11-Octadecenoic acid	(*cis*-Vaccenic acid)		-
cis-9,*trans*-11-Octadecadienoic acid	(*cis*-9,*trans*-11-Conjugated linoleic acid)		-
trans-10,*cis*-12-Octadecadienoic acid	(*trans*-10,*cis*-12-Conjugated linoleic acid)		-
Methyl *cis*-9,*cis*-12-octadecadienoate	(Methyl linoleate)		-
Monolinolein			-
Dilinolein			-
Trilinolein			-

537   [a], The activity of linoleic acid hydration (= 1.2 U/mg) under the condition (0.1 mM FAD,

538   5 mM NADH; 37°C, pH 5.5, 30 min) was defined as 100%. [b], not detected.

539

540 Table 2 **Substrate specificity of CLA-HY-catalyzed dehydration**

Substrate	Product		Relative activity [%]
10-Hydroxy-*cis*-12-octadecenoic acid	*cis*-9,*cis*-12-Octadecadienoic acid	(Linoleic acid)	100 [a]
10-Hydroxy-*cis*-12,*cis*-16-octadecadienoic acid	*cis*-9,*cis*-12,*cis*-15-Octadecatrienoic acid	(α-Linolenic acid)	127
10-Hydroxy-*cis*-6,*cis*-12-octadecadienoic acid	*cis*-6,*cis*-9,*cis*-12-Octadecatrienoic acid	(γ-Linolenic acid)	61
10-Hydroxyoctadecanoic acid	*cis*-9-Octadecenoic acid	(Oleic acid)	*tr.* [b]
12-Hydroxy-*cis*-9-octadecenoic acid			- [c]
12-Hydroxyoctadecanoic acid			-
3-Hydroxyhexadecanoic acid (C16)			-
9-Hydroxynonanoic acid (C9)			-

550 [a], The activity of 10-hydroxy-*cis*-12-octadecenoic acid dehydration (= 0.040 U/mg)

551 under the condition (0.1 mM FAD, 5 mM NADH; 37°C, pH 5.5, 15 min) was defined as

552 100%. [b]*tr.*, trace, <0.001%. [c], not detected.

553