

Linoleic Acid Isomerase in *Lactobacillus plantarum* AKU1009a Proved to Be a Multi-Component Enzyme System Requiring Oxidoreduction Cofactors

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Linoleic acid isomerase in *Lactobacillus plantarum* was found to be a novel multi-component enzyme system widespread in membrane and soluble fractions. The isomerization reaction involved a hydration step, 10-hydroxy-12-octadecenoic acid production from linoleic acid, as part of the reaction, and the hydration reaction was catalyzed by the membrane fraction. Both membrane and soluble fractions were required for the whole isomerization reaction, i.e., conjugated linoleic acid (CLA) production from linoleic acid, and for CLA production from 10-hydroxy-12-octadecenoic acid, a reaction intermediate. The multi-component enzyme system was inhibited by *o*-phenanthroline, and divalent metal ions such as Ni²⁺ and Co²⁺ restored activity. Metal oxides such as VO₄³⁺, MoO₄²⁺, and MnO₄²⁺ enhanced activity. The multi-component enzyme systems required oxidoreduction cofactors such as NADH together with FAD or NADPH for total activity.

Key words: conjugate linoleic acid (CLA); linoleic acid isomerase; *Lactobacillus plantarum*; lactic acid bacteria

Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) with conjugated double bonds. Individual isomers of CLA, e.g., *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoic acid, have been found to have potentially beneficial effects. For example, CLA prevents the initiation of carcinogenesis and tumorigenesis, reduces body fat content with simultaneous increases in muscle mass, controls immune systems, and alters the low-density lipoprotein/high-density lipoprotein cholesterol ratio.^{1–10)}

Natural sources of CLAs are dairy products from ruminants, although their CLA contents are very low. CLAs are formed as an intermediate by ruminal metabolism of polyunsaturated fatty acids by rumen bacteria.^{11,12)} Rumen bacteria are toxic to polyunsaturated fatty acids, so biohydrogenation occurs as the detoxification of polyunsaturated fatty acids to less toxic saturated fatty acid. Tove's group was the first to describe linoleic acid isomerase activity, producing *cis*-

9,*trans*-11-CLA from linoleic acid in *Butyrivibrio fibrisolvens*.¹³⁾ Although they clarified that the enzyme was associated with the membrane fraction, they could not purify the linoleic acid isomerase.¹⁴⁾ There are various reports on the enzymes involved in CLA production from linoleic acid by anaerobic bacteria. The *cis*-9,*trans*-11-CLA-producing isomerase from *Clostridium sporogenes* has been purified and characterized.¹⁵⁾ The enzyme was associated with the membrane fraction. The purified enzyme was a homotetramer, and the subunit molecular weight was 45 kDa. It required no external cofactors or energy sources for catalysis, and had a definite preference for toward substrates containing *cis* double bonds at the $\Delta 9$ and $\Delta 12$ positions of C18 polyunsaturated fatty acids.

As for the enzyme involved in *trans*-10,*cis*-12-CLA production, the enzyme from *Propionibacterium acnes*, which has been characterized as a polyunsaturated fatty acid isomerase,¹⁶⁾ was purified, cloned, expressed in *E. coli*, and crystallized. It was found to be a flavoprotein, and produced only *trans*-10,*cis*-12-CLA directly from linoleic acid, without forming any intermediate.

Many lactic acid bacteria, especially those belonging to the genus *Lactobacillus*, produce *cis*-9,*trans*-11-CLA as a major CLA isomer from linoleic acid. However, to the best of our knowledge, the enzymatic system of lactic acid bacteria involved in *cis*-9,*trans*-11-CLA production remains unclear. A candidate gene was reported, but the gene product expressed in *E. coli* did not produce any CLA isomers from linoleic acid, indicating that the enzyme needs extra factors for expression of activity.¹⁷⁾

We screened for the ability to produce CLA from linoleic acid within lactic acid bacteria, and selected *Lactobacillus plantarum* AKU 1009a as a potential strain. It produced two CLA isomers, *cis*-9,*trans*-11- and *trans*-9,*trans*-11-CLA, from linoleic acid with 10-hydroxy-12-octadecenoic acids as intermediates.^{18,19)} These results suggest that the reaction involves hydration and dehydrating isomerization catalyzed by multiple enzymes, while all reported linoleic acid isomerases produced CLA directly from linoleic acid without any intermediates. Hence we hypothesize that there are new

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Abbreviations: CLA, conjugated linoleic acid; CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; KPB, potassium phosphate buffer; US, supernatant after ultracentrifugation (soluble fraction); UPs, precipitates after ultracentrifugation (membrane fraction); EGTA, ethyleneglycol bis(2-aminoethylether) tetraacetic acid; HM, high-molecular-weight compounds; LM, low-molecular-weight compounds

reaction mechanisms of CLA production from linoleic acid in this strain.

This paper describes the enzymatic conversion of linoleic acid to CLA by fractions of cell-free extracts of *L. plantarum* AKU 1009a, and explains for the first time the involvement of multiple proteins and cofactors required for this activity.

Materials and Methods

Chemicals. Standard samples of *cis*-9,*trans*-11-octadecadienoic acid (CLA1), *trans*-9,*trans*-11-octadecadienoic acid (CLA2), and 10-hydroxy-*cis*-12-octadecenoic acid were prepared as described previously.^{18,20} These fatty acids in the reaction mixture were extracted, methyl-esterified, and isolated by reverse-phase high-performance liquid chromatography, and their chemical structures were identified by MS, and by ¹H-NMR. All NMR experiments were done with a Bruker Biospin DMX-750 (750 MHz for ¹H), and chemical shifts were assigned relative to the solvent signal. Fatty acid methyl esters were dissolved in CDCl₃ and analyzed by two-dimensional NMR techniques of ¹H-¹H double quantum filtered chemical shift correlation spectroscopy, ¹H clean-total correlation spectroscopy, and two-dimensional nuclear Overhauser effect spectroscopy.^{18,20} Linoleic acid and fatty acid-free (<0.02%) bovine serum albumin were purchased from Sigma (St. Louis, MO). All other chemicals used were of analytical grade and are commercially available.

Microorganism cultivation and preparation of washed cells. *L. plantarum* AKU 1009a (AKU Culture Collection, Faculty of Agriculture, Kyoto University) was cultivated in modified MRS medium (1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2% K₂HPO₄, 0.5% sodium acetate, 0.2% diammonium citrate, 0.02% MgSO₄·7H₂O, 0.005% MnSO₄·5H₂O, and 0.06% linoleic acid, pH 6.5). It was cultivated in 15 mL of medium in sealed screw-cap tubes (16.5 × 125 mm) at 28 °C for 24 h under O₂-limited conditions with shaking (120 strokes/min). The liquid medium occupied approximately 80 to 90% of the volume of the tubes. The seed culture (15 mL) was transferred to 550 mL of medium in a 600-mL Sakaguchi flask, followed by incubation at 28 °C for 24 h with shaking (120 strokes/min) to prepare a large number of cells. Grown cells were harvested by centrifugation (12,000 × g, 10 min), washed twice with 0.85% NaCl, and kept at -20 °C until use.

Preparation of cell-free extracts and fractionation. All operations were performed below 4 °C. Ten g (wet weight) of frozen cells were into 30 mL of 20 mM potassium phosphate buffer (KPB, pH 6.5), suspended, and treated with an ultrasonic oscillator (5 min, 4 times). Cell debris was removed by centrifugation at 1,700 × g for 10 min, and the supernatant was centrifuged again at 12,600 × g for 30 min. The resulting supernatant solutions were used as cell-free extracts, which were fractionated by ultracentrifugation at 100,000 × g for 90 min, and the supernatant solutions and precipitates were obtained. The supernatants after ultracentrifugation, the soluble fraction, was 30 mL of 20 mM KPB (pH 6.5) and used directly as the enzyme solution (US). The precipitates after ultracentrifugation, the membrane fraction, were 30 mL of 20 mM KPB (pH 6.5) and suspended, and the suspension was used as the enzyme solution (UP).

Reaction conditions. The reaction mixture, 1 mL (20 mM KPB, pH 6.5), in a test tube (16.5 × 125 mm) contained 0.4% w/v linoleic acid in a complex with BSA (0.08% w/v) or 0.3% w/v 10-hydroxy-*cis*-12-octadecenoic acid in a complex with BSA (0.06% w/v) as the substrate and 0.2 mL of enzyme solutions in various combinations of fractions. The reactions were carried out microaerobically in an O₂-adsorbed atmosphere in a sealed chamber with O₂ absorbent (Aneropack "Kenki"; Mitsubishi Gas Chemical, Tokyo), and gently shaken (120 strokes/min) at 37 °C for 24 h. Under microaerobic conditions, the oxygen concentration, monitored with an oxygen indicator (Mitsubishi Gas Chemical), was kept under 0.1% (<1,000 ppm). All experiments were carried out in triplicate, and the averages of three separate experiments that were reproducible within ± 10% are presented in the figures and tables.

Lipid analyses. *n*-Heptadecanoic acid was added to the reaction mixture before extraction as an internal standard. Lipids were extracted from 1 mL of the reaction mixture with 5 mL of chloroform-methanol-1.5% KCl in H₂O (2:2:1 by vol.) by the procedure of Bligh-Dyer and concentrated by evaporation under reduced pressure.²¹ The resulting lipids were dissolved in 1 mL of dichloromethane and then methylated with 2 mL of 4% methanolic HCl at 50 °C for 20 min. After the addition of 1 mL of water, the resulting fatty-acid methyl esters were extracted with 5 mL of *n*-hexane and 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 and a split injection system, and fitted with a capillary column (SPB-1, 30 m × 0.25 mm I.D.; SUPELCO, Bellefonte). The initial column temperature was 180 °C for 30 min, and this was raised to 210 °C at a rate of 60 °C/min and then maintained at 29.5 min. The injector and detector were operated at 250 °C. Helium was used as the carrier gas, at a flow rate of 1.4 mL/min. Peak identification of the fatty acids was carried out by comparison with the retention times of known standards.

Results

CLA productivity of enzyme fractions

Enzyme fractions were prepared by the procedures summarized in Fig. 1. Cell-free extracts before fractionation into the soluble fraction (US) and the membrane fraction (UP) by ultracentrifugation produced 0.21 mg/mL of CLA mixture (0.18 mg/mL of CLA1 and 0.03 mg/mL of CLA2) and 0.66 mg/mL of 10-hydroxy-*cis*-12-octadecenoic acid from linoleic acid; but fraction US only and fraction UP only did not transform linoleic acid to CLA (Table 1A). The UP fraction converted linoleic acid to 0.42 mg/mL of 10-hydroxy-*cis*-12-octadecenoic acid (Table 1A). Linoleic acid was transformed to CLA and 10-hydroxy-*cis*-12-octadecenoic acid in the presence of the US and UP fractions (Table 1A). In our previous study, 10-hydroxy-*cis*-12-octadecenoic acid was detected as an intermediate when *L. plantarum* AKU 1009a produced CLA from linoleic acid.^{22,23} When 10-hydroxy-*cis*-12-octadecenoic acid

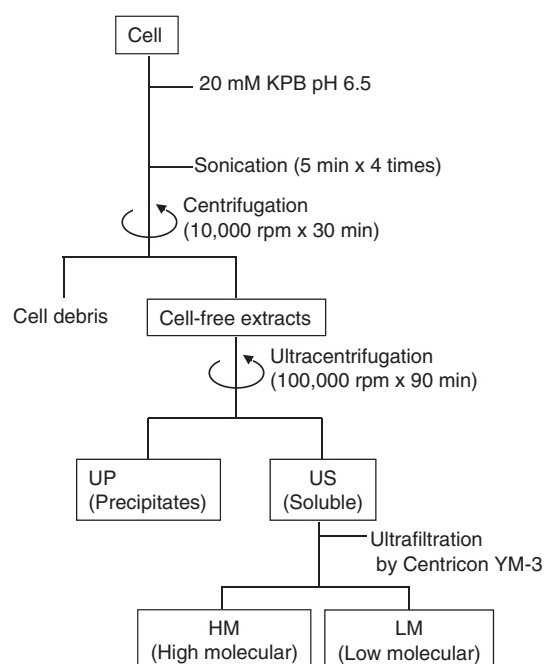


Fig. 1. Preparation of Enzyme Fractions from Cell-Free Extracts of *Lactobacillus plantarum* AKU 1009a.

Table 1. Catalytic Profiles of the Enzyme Fractions

A) Fatty acids produced from linoleic acid				
Fraction	Fatty acids produced (mg/mL)			
	LA	CLA1	CLA2	10-Hydroxy- <i>cis</i> -12-octadecenoic acid
Cell-free extracts	3.23	0.18	0.03	0.66
US	3.92	—	—	0.07
UP	3.09	—	—	0.42
US+UP	2.31	0.23	0.03	0.57

B) Fatty acids produced from 10-hydroxy- <i>cis</i> -12-octadecenoic acid				
Fraction	Fatty acids produced (mg/mL)			
	LA	CLA1	CLA2	10-Hydroxy- <i>cis</i> -12-octadecenoic acid
Cell-free extracts	0.21	0.68	0.18	0.86
US	0.05	0.17	0.03	1.42
UP	0.19	—	—	1.73
US+UP	0.19	0.72	0.19	0.74

The reaction mixture contained 0.4% w/v linoleic acid or 0.3% w/v 10-hydroxy-*cis*-12-octadecenoic acid as substrate, and 0.2 mL of enzyme solutions in various combinations of fractions. The reactions were carried out microaerobically at 37 °C for 24 h. LA, linoleic acid; CLA1, *cis*-9, *trans*-11-octadecadienoic acid; CLA2, *trans*-9, *trans*-11-octadecadienoic acid; US, soluble fraction; UP, membrane fraction.

was used as substrate, cell-free extracts produced 0.86 mg/mL of CLA mixture (0.68 mg/mL of CLA1 and 0.18 mg/mL of CLA2) and 0.21 mg/mL of linoleic acid (Table 1B). The US fraction produced small amounts of CLA and linoleic acid (Table 1B). The UP fraction transformed 10-hydroxy-*cis*-12-octadecenoic acid from linoleic acid. 10-Hydroxy-*cis*-12-octadecenoic acid was transformed into CLA in the presence of the US and UP fractions (Table 1). Based on these results, the enzymes involved in CLA production from linoleic acid in *L. plantarum* AKU 1009a were found to be separated into US and UP fractions. The UP fraction apparently hydrated linoleic acid to 10-hydroxy-*cis*-12-octadecenoic acid and dehydrated 10-hydroxy-*cis*-12-octadecenoic acid to linoleic acid. Although the US fraction did not show any significant catalytic activity by itself, the combination of US and UP resulted in expression of the activity of CLA production from linoleic acid and from 10-hydroxy-*cis*-12-octadecenoic acid.

Effects of chelating agents and metal ions on CLA production

The reaction was performed using cell-free extracts with chelating agents (3 mM). The chelating agents used were EDTA, ethyleneglycol bis(2-aminoethylether) tetraacetic acid (EGTA), 8-hydroxyquinoline, α - α' -bipyridyl, and *o*-phenanthroline. When EDTA, EGTA, 8-hydroxyquinoline, α - α' -bipyridyl, and *o*-phenanthroline were added to the reaction mixture, the CLA productivity of the cell-free extracts was 93%, 93%, 118%, 125%, and 10% respectively of that without chelating agents. We investigated the effects of metal ions and metal oxides (5 mM and 15 mM respectively) on CLA production by cell-free extracts in the presence of *o*-phenanthroline (3 mM), which are chelates of transition elements. Metal ions, such as Co²⁺, Ni²⁺, Fe³⁺, Cd³⁺,

Table 2. Effects of Divalent Metal Ions and Metal Oxides on CLA Production of the Multi-Component Enzyme System in the Presence of *o*-Phenanthroline

Relative productivity (%)	
Control (without <i>o</i> -phenanthroline)	100
Metal addition with <i>o</i> -phenanthroline	
None	11
CuSO ₄	0
CeSO ₄	0
ZnSO ₄	10
MgSO ₄	10
MnSO ₄	21
CdCl ₂	29
FeSO ₄	31
NiCl ₂	41
CoCl ₂	56
VO	0
V ₂ O ₃	0
V ₂ O ₅	0
VO ₂	5
NaVO ₃	209
Na ₃ VO ₄	264
H ₂ WO ₄	25
K ₂ WO ₄	149
Na ₂ WO ₄	157
MoO ₃	8
MoO ₂	13
K ₂ MoO ₄	422
Na ₂ MnO ₄	427

The reaction mixture contained 0.4% w/v linoleic acid, 0.2 mL of cell-free extracts, 3 mM *o*-phenanthroline, and metal compounds. The reactions were carried out microaerobically at 37 °C for 24 h. A control reaction was done without metal compounds or *o*-phenanthroline. The concentrations of the divalent metal ions and metal oxides were 10 mM and 5 mM respectively. One hundred percent of the control reaction corresponded to 0.21 mg/mL of CLA.

and Mn²⁺, restored CLA productivity to some extent, and metal oxides, such as MnO₄²⁻, MoO₄²⁻, VO₄³⁻, VO₃⁻, and WO₄²⁻, enhanced CLA productivity to a maximum of 400% of the original activity (Table 2).

Factors affecting CLA production

A 15-mL US fraction was ultrafiltered using an Amicon ultracentrifugal filter unit fitted with a Centri-con YM-3 membrane with a 3,000-Da nominal molecular weight cutoff (Millipore, Billerica). The retained fraction with high molecular weight compounds (>3,000 Da) was named HM and the fraction with low molecular weight compounds (<3,000 Da) was named LM. HM and LM were 15 mL of 20 mM KPB (pH 6.5) and used as enzyme solutions. When all the HM, LM, and UP fractions were combined, 108% of the original CLA-producing activity (the activity of the US and UP fractions) was obtained, and 67% of the original CLA-producing activity when HM and UP fractions were used together, and no activity when the LM and the UP fraction were used together. As described above, the LM fraction was required to reproduce the CLA-producing activity completely. This result indicates that the LM fraction contains low-molecular-cofactor compounds that are necessary for the original CLA-producing activity. To identify which cofactor is effective for the reaction, we added some known cofactors to the reaction mixture. CLA productivity with a dialyzed HM fraction in combination with the UP fraction was about 50% of

Table 3. Effects of Redox Cofactors on the Linoleic Acid Isomerizing Activity of the Multi-Component Enzyme System

Cofactor	Relative productivity (%)
US+UP	100*
Cofactor addition with US and HM	
None	46
NAD ⁺	16
NADH	43
NADP ⁺	20
NADPH	98
FMN	27
FAD	30
FMN+NADH	50
FAD+NADH	101
FMN+NADPH	36
FAD+NADPH	11

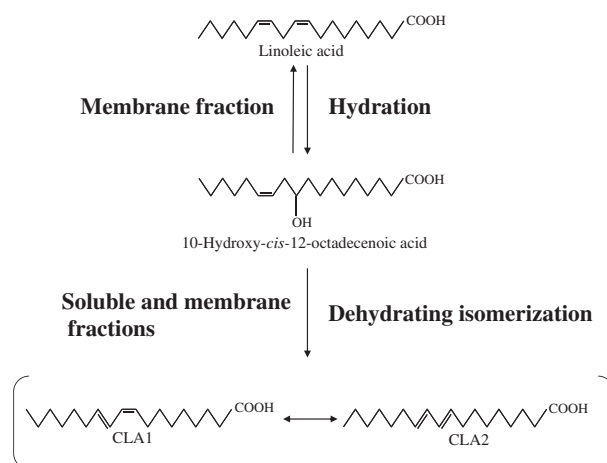
The reaction mixture contained 0.4% w/v linoleic acid, 0.2 mL of the membrane fraction (UP), and 0.2 mL of the dialyzed high molecular fraction (HM) of the soluble fraction (US), and 5 mM redox cofactors, except for "None." The reactions were carried out microaerobically at 37 °C for 24 h.

*CLA productivity with the US fraction in combination with the UP fraction (0.21 mg/mL of CLA) was defined as 100%.

that with the US and UP fractions. Among the cofactors tested, some redox cofactors were found to enhance activity. In particular, the addition of NADPH or FAD and NADH to the reaction mixture with dialyzed HM restored activity (Table 3).

Discussion

In our previous study, we found that CLA production from linoleic acid by *L. plantarum* AKU 1009a consists of two steps, hydration of linoleic acid to 10-hydroxy-12-octadecenoic acids and dehydrating isomerization of 10-hydroxy-12-octadecenoic acids to CLA (Fig. 2). In the present study, hydrating and dehydrating activities were found in the membrane fraction, while CLA production from linoleic acid and from 10-hydroxy-*cis*-12-octadecenoic acid was detected only in the presence of both soluble and membrane fractions (Fig. 2). By the combined action of the soluble and membrane fractions, CLA production from 10-hydroxy-*cis*-12-octadecenoic acid proceeded more efficiently than from linoleic acid. These findings suggest that hydration of linoleic acid by the membrane fraction is a rate-limiting step in CLA production from linoleic acid. CLA production by the enzyme systems of this strain was strongly inhibited by a chelating agent, *o*-phenanthroline, and activity was restored by certain divalent metal ions, and was further enhanced by metal oxides, including MnO₄²⁻ and MoO₄²⁻, similarly to anaerobic enzymes, including 4-hydroxybenzoyl-CoA reductase, pyrogallol-phloroglucinol transhydroxylase, and acetylene hydratase activated by these metal oxides, as reported earlier.²⁴⁾ Furthermore, this reaction required NADPH or NADH and FAD as cofactors for full activity. The functions of these oxidoreduction cofactors and metal ions in the linoleic acid isomerase system remain unknown. However, there are two possibilities: (a) oxidative and/or reductive modifications of the hydroxyl group in the intermediate, 10-hydroxy-*cis*-12-octadecenoic acid, requiring NAD(P)H are involved in the whole conversion process, and (b) NAD(P)H is necessary for the reduction of FAD.

**Fig. 2.** Proposed Pathway of CLA Production from Linoleic Acid Catalyzed by the Multi-Component Enzyme System in *Lactobacillus plantarum* AKU1009a.

CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid.

Many studies of microbial CLA production with *Clostridium*, *Butyrivibrio*, *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Megasphaera*, and other rumen bacteria have reported that linoleic acid was transformed to *cis*-9,*trans*-11- or/and *trans*-10,*cis*-12-CLA by a single enzyme, linoleic acid isomerase, without generating any intermediates.^{13–15,25–27)} Most of these bacterial isomerases were found in the membrane fractions. As for *Propionibacterium acnes*, polyunsaturated fatty acid isomerase was cloned, expressed in *E. coli* BL21 (DE3), and crystallized. This enzyme contains FAD and requires no external cofactors or energy sources for catalysis. As for *Clostridium sporogenes*, the linoleic acid isomerase of this strain was membrane-associated and required no external cofactor or energy source for catalysis.

From these findings, we deduce that the reaction mechanism of CLA production from linoleic acid by *L. plantarum* and the enzyme systems involved differ from those reported for other strains. Further analysis of the multi-component enzyme system through protein purification and gene identification is ongoing.

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