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*Note*

# Ricinoleic Acid and Castor Oil as Substrates for Conjugated Linoleic Acid Production by Washed Cells of *Lactobacillus plantarum*

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**Ricinoleic acid (12-hydroxy-*cis*-9-octadecaenoic acid) was an effective substrate for conjugated linoleic acid (CLA) production by washed cells of *Lactobacillus plantarum* AKU 1009a. The CLA produced was a mixture of *cis*-9,*trans*-11- and *trans*-9,*trans*-11-octadecadienoic acids. Addition of α-linolenic acid to the culture medium increased the CLA productivity of the washed cells. In the presence of lipase, castor oil, in which the main fatty acid component is ricinoleic acid, also was a substrate for CLA.**

**Key words:** conjugated linoleic acid; ricinoleic acid; castor oil; lactic acid bacteria; *Lactobacillus plantarum*

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Conjugated linoleic acid (CLA) is a collective term for isomers of linoleic acid with conjugated double bonds. Specific CLA isomers such as *cis*-9,*trans*-11-octadecadienoic acid (18:2) and *trans*-10,*cis*-12-18:2 may have beneficial physiological and anticarcinogenic effects.[1–4] In this paper, we present the first example of the biosynthesis of CLA from ricinoleic acid and castor oil. Our previous studies showed that two CLA isomers, CLA1 [*cis*-9,*trans*-11-octadecadienoic acid (18:2)] and CLA2 (*trans*-9,*trans*-11-18:2) were efficiently produced from linoleic acid on incubation with washed cells of lactic acid bacteria. (In a previous paper, CLA1 was identified as *cis*-9,*trans*-11- or *trans*-9,*cis*-11-18:2; however, we recently confirmed the exact structure as *cis*-9,*trans*-11-18:2 based on detailed 2-dimensional NMR analysis; Kishino *et al*. unpublished results) Analysis of the pathway of CLA production from linoleic acid by lactic acid bacteria indicated the participation of two hydroxy fatty acids, 10-hydroxy-*cis*- and 10-hydroxy-*trans*-12-octadecaenoic acid (18:1), as possible intermediates.[5]

In this study, we evaluated a hydroxy fatty acid, ricinoleic acid (12-hydroxy-*cis*-9-18:1), the chemical structure of which is similar to that of 10-hydroxy-12-18:1, as an alternative substrate for CLA production by the lactic acid bacterium *Lactobacillus plantarum*. Ricinoleic acid is readily available from castor oil and would be a practical substrate for microbial CLA production.

Washed cells of *L. plantarum* AKU 1009a[6] (AKU culture collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan), selected as a potential catalyst for CLA production from linoleic acid *via* hydroxy fatty acids, were given a preliminary examination. Cultivation, reactions, and analyses were done under the conditions described previously.[5,6] *L. plantarum* AKU 1009a was cultivated in MRS medium (10 g of Polypepton [Nihon-pharm. Co., Tokyo, Japan], 10 g of meat extract [Mikuni Co., Tokyo], 5 g of yeast extract [Difco, Sparks, MD], 20 g of glucose, 1 g of Tween 80, 2 g of $K_2HPO_4$, 5 g of sodium acetate, 2 g of diammonium citrate, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $MnSO_4 \cdot 5H_2O$ in 1 liter, pH 6.5 by NaOH) containing various fatty acids (0.6 g/l as free fatty acids). After cultivation in 550 ml of liquid medium in 600-ml flasks for 24 h at 28°C with shaking (120 strokes/min), the cells were harvested by centrifugation (8,000 × *g*, 10 min) and used. The reactions were done microaerobically in an $O_2$-adsorbed atmosphere in a sealed chamber with $O_2$-absorbent (Aneropack ''Kenki'', Mitsubishi Gas Chemical Co., Ltd., Tokyo), and gently shaken (120 strokes/min) at 37°C for 24 h. The reaction mixture, 1 ml, in a test tube (16.5 × 125 mm) contained 4.0 mg/ml ricinoleic acid or linoleic acid mixed with 0.8 mg/ml bovine serum albumin (BSA, fatty-acid-free, Sigma, St. Louis, MO) to increase the solubility of the fatty acid, 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells (corresponding to 3.2% [dry cells, w/v]) cultivated with various fatty acids.

As the washed cells of lactic acid bacteria cultivated in medium containing 0.6 g/l linoleic acid produced much CLA from linoleic acid,[5] the washed cells of *L. plantarum* cultivated in the MRS medium containing various fatty acids were evaluated for CLA productivity from ricinoleic acid or linoleic acid. Among the fatty acids tested as additives (0.6 g/l) in the medium (linoleic acid, α-linolenic acid, oleic acid, ricinoleic acid, and castor oil), α-linolenic acid

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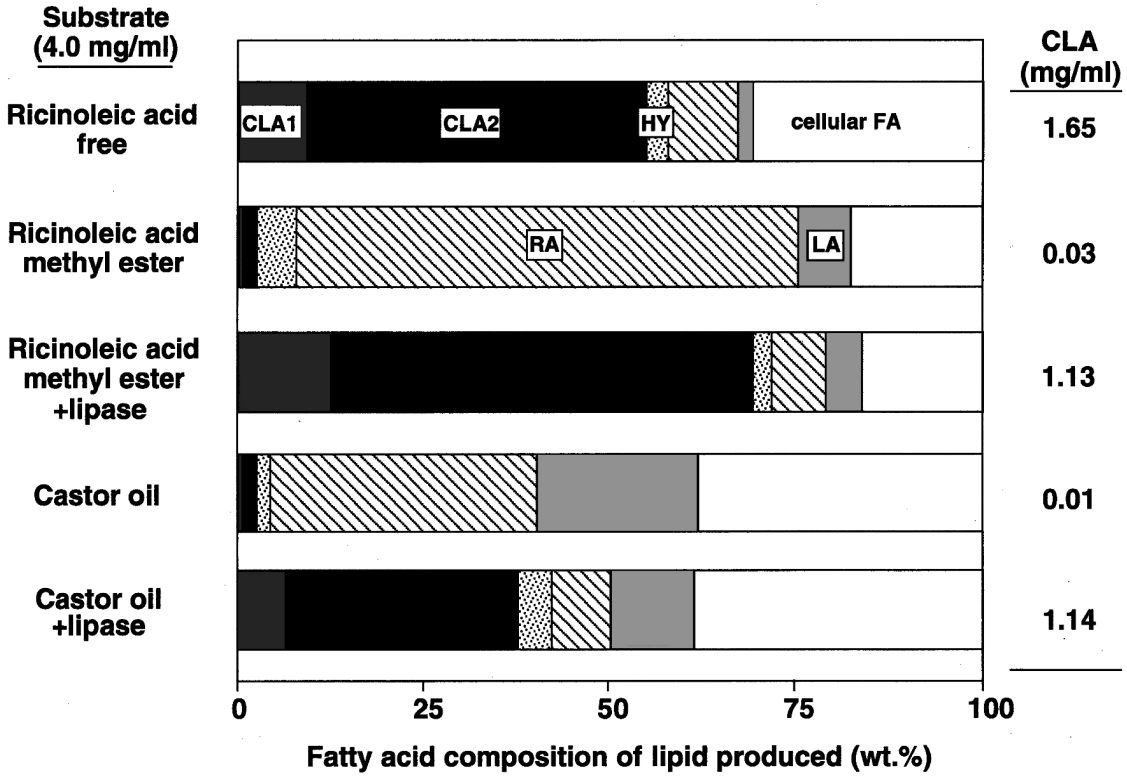


**Fig. 1.** CLA Production from Different Forms of Ricinoleic Acid and Castor Oil with or without Lipase (Lipase M "Amano" 10).
The reactions were done with washed cells of *L. plantarum* 1009a as the catalyst. The washed cells were obtained by centrifugation after cultivation in MRS medium containing 0.6 g/l α-linolenic acid. All experiments were done in triplicate, and the means of three separate experiments (reproducible within ±10%) are given. CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; HY, 10-hydroxy-*cis*-12-octadecaenoic acid and 10-hydroxy-*trans*-12-octadecaenoic acid; RA, ricinoleic acid; LA, linoleic acid. Cellular fatty acid (cellular FA): myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid.

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increased CLA production from ricinoleic acid to 1.04 mg/ml (molar conversion yield, 26%). The CLA produced was identified as a mixture of CLA1 (0.28 mg/ml) and CLA2 (0.76 mg/ml). Linoleic acid (0.6 g/l), which increases the CLA production from linoleic acid, was not effective on the CLA production from ricinoleic acid (CLA production, 0.04 mg/ml), and the combination of linoleic acid (0.6 g/l) and α-linolenic acid (0.6 g/l) also decreased the CLA production from ricinoleic acid (CLA production, 0.58 mg/ml).

The changes in CLA productivity during cultivation in the medium with 0.6 g/l α-linolenic acid were investigated. The cells after 48 h of cultivation (mid-logarithmic phase; $OD_{610}$, 3.25) produced 1.52 μg of CLA per mg of dry cells per hour, but prolonged cultivation did not further increase the productivity. Washed cells obtained after 48 h of cultivation were used for further experiments.

Free and methyl ester forms of ricinoleic acid and castor oil, in which the major fatty acid component is ricinoleic acid, were tested as substrates (4.0 mg/ml) for CLA production after they were mixed with 0.8 mg/ml BSA. Castor oil (triacylglycerol of fatty acids [ricinoleic acid 88.2%, linoleic acid 4.8%, and other 7.0%]) was obtained from Itoh Oil Chemicals Co. (Yokkaichi, Japan). Reactions were done as described above except for the substrates. The free form of ricinoleic acid was converted to CLA at 1.65 mg/ml, but little CLA was produced from the methyl ester or castor oil (Fig. 1).

Castor oil could be the substrate if the lipases converted it to the free form, ricinoleic acid. Eight lipases obtained from Amano Enzyme Co. (Nagoya, Japan) (Bioenzyme M, Lipase AH-S, Lipase GC "Amano" 4, Lipase PS-C "Amano" I, Pancreatin F, Lipase AY "Amano" 30, Lipase F-AP 15, and Lipase M "Amano" 10) were tested for their ability to produce free-form ricinoleic acid from castor oil. Production of free-form ricinoleic acid from castor oil by lipase was monitored in reaction mixtures containing 4.0 mg/ml castor oil, 0.1 M potassium phosphate buffer, pH 6.5, and 100 U/ml each lipase. Reactions were done at 37°C for 24 h and the reaction mixtures were analyzed by TLC on silica gel 60 $F_{254}$ (Merck, Darmstadt, Germany) with *n*-hexane-diethyl ether-acetic acid (60:40:1, [v/v]) and 5% 12-molybdo(VI)phosphoric acid *n*-hydrate in ethanol as the developing solvent and detection reagent, respectively. TLC showed that Lipase M "Amano" 10 pro-

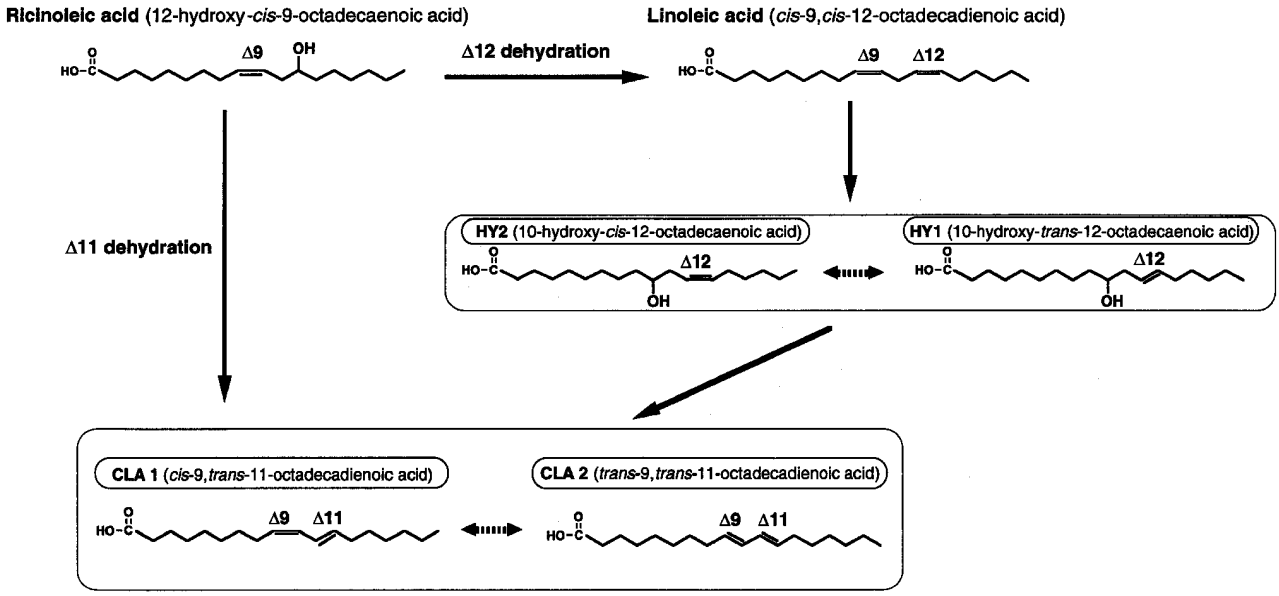


**Fig. 2.** Proposed Pathway of CLA Production from Ricinoleic Acid by *L. plantarum*.

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duced the most free-form ricinoleic acid (data not shown). To produce CLA from castor oil by the washed cells of *L. plantarum*, reactions were done with 4.0 mg/ml castor oil in the presence of Lipase M "Amano" 10 (100 U/ml). As shown at the bottom of Fig. 1, 1.14 mg/ml CLA was produced from castor oil in the presence of the lipase (molar conversion yield, 28.5%). The CLA produced was a mixture of CLA1 (0.19 mg/ml) and CLA2 (0.95 mg/ml). Ricinoleic acid methyl ester also became an efficient substrate in the presence of lipase (Fig. 1).

Chemical syntheses of CLA from ricinoleic acid and castor oil were reported previously.[7,8] We presented here the biosynthesis of CLA from ricinoleic acid and castor oil. Although only the free form of ricinoleic acid is a suitable substrate for this reaction, CLA was produced from castor oil also with the help of lipases. These results suggest the possibility of development of a new process for CLA production from readily available castor oil with *L. plantarum*.

There are two possible pathways for CLA synthesis from ricinoleic acid by *L. plantarum*; i) direct transformation of ricinoleic acid to CLA through dehydration at the $\Delta 11$ position, and ii) dehydration of ricinoleic acid at the $\Delta 12$ position to linoleic acid, which is a potential substrate for CLA production by lactic acid bacteria (Fig. 2). The observation that the cells cultivated in the medium containing $\alpha$-linolenic acid produced much CLA from ricinoleic acid, although those cultivated in the medium containing linoleic acid produced little, suggested the significance of the former pathway. However, the existence of linoleic acid and 10-hydroxy-12-18:1 in the reaction mixture with ricinoleic acid as a substrate also indicated the participation of the latter pathway.

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