Note

Structural Analysis of Conjugated Linoleic Acid Produced by Lactobacillus plantarum, and Factors Affecting Isomer Production

Shigenobu KISHINO,¹ Jun OGAWA,¹ Akinori ANDO,¹ Takashi IWASHITA,² Tsuyoshi FUJITA,² Hiroshi KAWASHIMA,³ and Sakayu SHIMIZU¹,⁹

¹Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
²Suntory Institute for Bioorganic Research, Shimamoto-cho, Mishima-gun, Osaka 618-8503, Japan
³Institute for Fundamental Research, Suntory Ltd., Shimamoto-cho, Mishima-gun, Osaka 618-0001, Japan

Received June 18, 2002; Accepted September 26, 2002

An isomer of the conjugated linoleic acid (CLA) produced from linoleic acid by Lactobacillus plantarum was identified as cis-9,trans-11-octadecadienoic acid by proton nuclear magnetic resonance spectroscopy. Together with earlier results, we concluded that the bacterium produces two CLA isomers, cis-9,trans-11- and trans-9,trans-11-octadecadienoic acid from linoleic acid. The addition of L-serine, glucose, AgNO₃, or NaCl to the reaction mixture reduced production of the latter.

Key words: conjugated linoleic acid; linoleic acid; lactic acid bacteria; Lactobacillus plantarum

Conjugated linoleic acid (CLA), an octadecadienoic acid with conjugated double bonds, has a variety of positional and geometric isomers. Among these isomers, cis-9,trans-11-octadecadienoic acid (18:2) and trans-10,cis-12-18:2 have attracted attention because of their unique physiological effects such as inhibition of carcinogenesis and reduction of the body fat content.¹⁻⁴ These isomers act both independently and together to produce the multitude of physiological effects that attribute to CLA. Decreased body fat gain is an example of a single-isomer effect caused by trans-10,cis-12-18:2. Various specific effects of isomers are being identified in a number of laboratories. We found that washed cells of lactic acid bacteria produce two CLA isomers from linoleic acid and from ricinoleic acid,⁵,⁶ and established practical processes for CLA production with washed cells of Lactobacillus plantarum AKU 1009a as the substrate and catalyst, respectively. Fatty acids in the reaction mixtures were extracted, methyl-esterified, and isolated by high-pressure liquid chromatography and their chemical structures were identified by MS as described previously,⁵ 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 (DQF-COSY), ¹H clean-total correlation spectroscopy (clean-TOCSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY).

The isolated CLA1 methyl ester was transformed into a CLA1 pyrrolidine derivative.⁹ The mass spectrum of the pyrrolidine derivative showed a molecular weight at m/z 333. This result suggested that CLA1 was a C₁₈ fatty acid containing two double bonds. The FAB-MS data for the free fatty acid of CLA¹³ showed a molecular weight at 280 (m/z 279 [M–H]⁺). The material (m/z 279) was fragmented again by MS-MS [m/z (FAB⁺, 8.00 kV), 263 (6), 249 (5), 235 (5), 221 (16), 208 (11), 207 (11), 193 (5), 181 (4), 167 (3), 141 (12), 127 (71), 113 (10), 100 (8), 98

To whom correspondence should be addressed. Tel: +81-75-753-6115; Fax: +81-75-753-6128; E-mail: sim@kais.kyoto-u.ac.jp

Abbreviation: CLA, conjugated linoleic acid
Fig. 1. $^1$H NMR Analysis of CLA1 and Structure of CLA1 Identified.
(A) Structure of CLA1. (B) $^1$H clean-total correlation spectroscopic spectrum of the methyl ester of CLA1. (C) Two-dimensional nuclear Overhauser effect spectroscopy of the methyl ester of CLA1.

(10), 86 (18), 72 (11), 71 (57), 58 (100)]. Typical fragments ($m/z$) for CLA1 were 127, 141, 167, 193, 207, and 208. The $m/z$ 141, 167 and 193 fragments were derived through cleavage at single bonds 8–9, 10–11, and 12–13, as numbered from the carboxyl group. The $m/z$ 127 and 207 fragments, derived through the cleavage of the single bond between the $\alpha$ and $\beta$ positions from the double bond, were detected clearly. Hence, CLA1 was identified as a 9,11 positional isomer of octadecadienoic acid. Results of IR analysis of CLA1 methyl esters were as follows: IR $\nu_{max}$ (CHCl$_3$) cm$^{-1}$: 2923, 2846, 1742, 1461, 1435, 1196, 1170, 990, 944. The peaks at 990 and 944 indicated that CLA1 was a cis/trans isomer. $^1$H NMR analysis also suggested that CLA1 was an isomer of 18:2 (Fig. 1) [NMR $\delta_H$ (CDCl$_3$): 6.29 (1H, dd, $J=15.0$, 10.5 Hz, =CH–CH=), 5.94 (1H, dd, $J=11.2$, 10.5 Hz, =CH=CH=), 5.66 (1H, dt, $J=15.8$, 6.8 Hz, –CH=CH=), 5.29 (1H, dt, $J=10.5$, 7.5 Hz, –CH=CH=), 3.67 (3H, s, –OC$_3$H$_7$), 2.30 (2H, t, $J=7.5$ Hz, –COCH$_2$–), 2.14 (2H, dt, $J=7.5$, 6.8 Hz, –CH$_2$–CH=), 2.09 (2H, dt, $J=7.5$, 6.8 Hz, =CH–CH=), 1.62 (2H, m, –CH$_2$CH=CH$_2$–), 1.39 (4H, m, –CH$_2$CH=CH$_2$–), 1.30 (12H, m, –CH$_2$CH=CH$_2$–), 0.88 (3H, t, $J=7.1$ Hz, –CH$_3$)]. Signals G (5.29 ppm), H (5.66 ppm), I (5.94 ppm), and J (6.29 ppm) indicated the existence of two double bonds in CLA1 (Fig. 1). The sequence of the protons from the methyl end of the molecule was deduced to be A, B, C, H, J, I, G, D, B, and E or A, B, D, G, I, J, H, C, B, and E on the basis of the signal pattern of the interaction between adjacent protons observed by DQF-COSY. The sequence was confirmed to be the former one by the appearance of an interaction signal between A and C but not A and D on clean-TOCSY analysis (Fig. 1(B)), indicating that C was near A, but that D was far from A.

NOESY was done to identify the geometric configurations of double bonds. The appearance of a cross-peak between D and J suggested that the double bond between G and I had the cis configuration (close enough to interact) (Fig. 1(C)). This conclusion was confirmed by analysis of the spin-spin coupling constants between the G and I protons (11.2 and 10.5 Hz); the spin-spin coupling constants between the J and H protons (15.0 and 15.8 Hz) suggested that the double bond between J and H was in the trans configuration. On the basis of the results of spectral analyses, CLA1 was identified as cis-9,trans-11-18:2 (Fig. 1(A)). These results together with previous results showed that the bacterium produced two CLA isomers, cis-9,trans-11- and trans-9,trans-11-
CLA Isomers Produced by *Lactobacillus plantarum*

It is important to produce the CLA isomers selectively for evaluation of their biological and physiological effects. CLA2 can be produced at more than 97% purity by *L. plantarum* AKU 1009a, if the reaction is done long enough with a low linoleic acid concentration. However, selective production of CLA1 has never been achieved, whatever the reaction conditions. Hence, the effects of various compounds on isomer production were investigated. Among nine sugars (10%, w/v), 37 amino acids (1%, w/v), 31 metal ions (1 to 10 mM), 10 salts (10%, w/v), four enzyme cofactors (40 mM), and 43 enzyme inhibitors (1 to 10 mM) added one by one to the reaction mixture with linoleic acid and washed cells of *L. plantarum* as the substrate and catalysts, respectively, the several compounds listed in Table 1 affected isomer production. These compounds reduced CLA2 production, by which the apparent selectivity for CLA1 was increased. Effects of the concentration of L-serine, glucose, NaCl, and AgNO₃ were examined (Fig. 2). Additions of 5 to 10% (w/v) L-serine or 5 to 10 mM AgNO₃ were effective for selective production of CLA1. Interestingly, D-serine did not have such an effect. The additions of 5 to 15% (w/v) NaCl or 5 to 10 mM AgNO₃ slightly increased CLA1 production and reduced CLA2 production. Above all, the production of CLA1 and CLA2 were independently controlled by these compounds. These results indicated that there were different pathways for biosynthesis of these isomers. Lactic acid bacteria cannot synthesize these isomers de novo, although they transform exogenous linoleic acid to CLA isomers for detoxification of free polyunsaturated fatty acids.

**Fig. 2.** Effects of Concentrations of L-Serine (A), Glucose (B), NaCl (C), and AgNO₃ (D) on CLA Isomer Production.

Reactions were done as described previously⁷ with 4.0 mg/ml linoleic acid and 22.5% (w/v) wet washed cells of *L. plantarum* AKU 1009a as the substrate and catalysts, respectively, at 37°C for 24 h, except for the addition of the indicated concentrations of a compound. Fatty acids in the reaction mixtures were extracted, methyl-esterified, and analyzed on gas-liquid chromatography as described previously.⁵

**Table 1.** Effects of Various Compounds on CLA Isomer Production

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Fatty acid (mg/ml of reaction mixture)</th>
<th>CLA1</th>
<th>CLA2</th>
<th>Total CLA</th>
<th>CLA1/CLA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>0.32</td>
<td>1.89</td>
<td>2.21</td>
<td>0.17</td>
</tr>
<tr>
<td>Amino acid</td>
<td></td>
<td></td>
<td>0.34</td>
<td>0.30</td>
<td>0.64</td>
<td>1.13</td>
</tr>
<tr>
<td>L-Serine</td>
<td>1% (w/v)</td>
<td></td>
<td>0.23</td>
<td>0.23</td>
<td>0.46</td>
<td>1.00</td>
</tr>
<tr>
<td>Sugar</td>
<td></td>
<td></td>
<td>0.28</td>
<td>0.37</td>
<td>0.65</td>
<td>0.76</td>
</tr>
<tr>
<td>Glucose</td>
<td>10% (w/v)</td>
<td></td>
<td>0.33</td>
<td>0.50</td>
<td>0.83</td>
<td>0.66</td>
</tr>
<tr>
<td>Maltose</td>
<td>10% (w/v)</td>
<td></td>
<td>0.45</td>
<td>0.96</td>
<td>1.41</td>
<td>0.47</td>
</tr>
<tr>
<td>Fructose</td>
<td>10% (w/v)</td>
<td></td>
<td>0.45</td>
<td>0.15</td>
<td>0.62</td>
<td>3.13</td>
</tr>
<tr>
<td>Salt</td>
<td></td>
<td></td>
<td>0.34</td>
<td>0.10</td>
<td>0.44</td>
<td>3.40</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 mM</td>
<td></td>
<td>0.45</td>
<td>0.23</td>
<td>0.68</td>
<td>1.96</td>
</tr>
<tr>
<td>Metal</td>
<td>2,3,5-Triphenyltetrazolium chloride</td>
<td>10 mM</td>
<td>0.45</td>
<td>0.30</td>
<td>0.75</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Reactions were done as described previously⁷ with 4.0 mg/ml linoleic acid and 22.5% (w/v) wet washed cells of *L. plantarum* AKU 1009a as the substrate and catalysts, respectively, at 37°C for 24 h, except for the addition of the indicated concentrations of a compound. Control experiments were done without additions. Fatty acids in the reaction mixtures were extracted, methyl-esterified, and analyzed by gas-liquid chromatography as described previously.⁵

Acknowledgments

This work was partially supported by Grants-in-
Aid for Scientific Research (No. 13853009 and No. 13876023 to S. S.) from the Ministry of Education, Science, Sports, and Culture, Japan. S. K. is a recipient of a Research Fellowship (No. 01985) of the Japan Society for the Promotion of Science for Young Scientists.

References