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Production of conjugated linoleic acid by enzyme extract of *Lactobacillus acidophilus* CCRC 14079

T.Y. Lin[a,*], C.-W. Lin[b], Y.-J. Wang[b]

[a]*Department of Animal Science, Chinese Culture University, #55 Hwa Kang Road., Yang Ming Shan, Taipei 111, Taiwan*
[b]*Department of Animal Science, National Taiwan University, Taipei, Taiwan*

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Abstract

Crude enzyme extracted from *Lactobacillus acidophilus* (CCRC 14079) was tested for the production of conjugated linoleic acids (CLA) reported to have anticarcinogenic, antiatherosclerotic, antidiabetogenic, antiobese, and immunomodulative properties. Levels of 50 and 75 mg linoleic acid (LA) were reacted with 25, 50, and 75 mg enzyme extract at 50 °C for 10 min at pH 5, and the amounts of CLA produced were determined by high performance liquid chromatography. The sharp increases in total CLA levels from 8 to 305 μg in 50 mg LA treatment and from 116 to 439 μg in 75 mg LA treatment were observed as enzyme extract level increased from 0 to 50 mg. Total CLA level remained unchanged as enzyme level increased to 75 mg. The average percentages of CLA isomers in total CLA produced in the reactions of enzyme extract and LA ranged from 6 to 19%. Forty eight % of those isomers were in *cis*,*trans*/*trans*,*cis* form and 14% were c9,t11-CLA.

Keywords: Conjugated linoleic acid; Linoleic acid isomerase; *Lactobacillus acidophilus*

1. Introduction

Conjugated linoleic acids (CLA), a naturally occurring group of positional and geometric isomers of octadecadienoic fatty acids with conjugated double bonds, have been reported in animals to have anticarcinogenic (Belury, 1995; Kimoto et al., 2001; Parodi, 1996; Scimeca, Thermopson, & Ip, 1994), antiatherosclerotic (Lee, Kritchevsky, & Pariza, 1994; Nicolosi, Rogers, Kritchevsky, Scimeca, & Huth, 1997), antidiabetogenic (Houseknecht et al., 1998), antiobese and body mass-enhancing (Park et al., 1999; West et al., 1998), antioxidative (Decker, 1995), immunomodulative (Hayek et al., 1999), antibacterial (Sugano et al., 1997), cholesterol depressing (Huang, Kuedecke, & Shultz, 1994), and growth promoting (Chin, Storvkson, Liu, Albright, & Pariza, 1994) properties.

Different isoforms may have different biological actions. For example, c9,t11-CLA, the principal biologically active isomer (Ha & Lindsay, 1990; Ip, Chin, Scimeca, & Pariza, 1991) in foods (Pariza, Park, & Cook, 2001), was thought to be active as a potential antioxidant and anticarcinogenic agent (Lin, Boylston, Chang, Luedecke, & Schults, 1995). Similarly, t10,c12- and t8,c10-CLA may be responsible for body fat reduction and lean body mass enhancement. (Baumgard, Corl, Dwyer, Saebo, & Bauman, 2000; Chouinard, Corneau, Saebo, & Bauman, 1999; Park et al., 1999).

CLA isomers are found predominantly in foods originating from ruminants (Chin, Liu, Storkson, Ha, & Pariza, 1992; Chin et al., 1994). The formation of this conjugated polyunsaturated fatty acid was catalyzed by ruminal bacteria (Barlett & Chapman, 1961; Chin et al., 1994; Shorland, Weenink, Johns et al., 1995). *Butyrivibrio fibrisolvens*, the most widely known organism in rumen (Jenkins, 1993; Lal & Narayanan, 1984), was shown to be capable of CLA formation in the ruminal biohydrogenation of linoleic acid (LA) by the action of LA isomerase (Kepler, Hirons, McNeill, & Tove, 1966; Kepler, Tucke, & Tove, 1970, 1971). Successful separation and purification of LA isomerase from *B. fibrisolvens* by Chin et al. (1992, 1994) further demonstrated the catalyzing action of this bacterial enzyme on CLA formation.

* Corresponding author. Tel.: +8862-2861-0511x503; fax: +8862-2861-3100.
E-mail address: tylin@faculty.pccu.edu.tw (T.Y. Lin).

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LA isomerase activity was also observed in the unknown bacterial strains of rat's intestine (Yang & Pariza, 1995), microorganism-containing feeds (Chin et al., 1994), and intestinal bacteria of humans (Kamlage, Hartmann, Gruhl, & Blaut, 2000). More recently, strains of propionibacteria (Jiang, Bjröck, & Fondén, 1998) and lactic acid bacteria (Lin, 2000; Lin, Lin, & Lee, 1999) were identified to be capable of isomerizing LA into CLA in vitro through LA isomerase (Lin, Lin, & Wang, 2002).

In the previous study, higher LA isomerase activity was observed in the enzyme extracts of *L. acidophilus* than *Propionibacterium freudenreichii* ssp. *shermanii*, and the optimal reaction pH was pH 5 (Lin et al., 2002). In order to further examine the effect of various levels of enzyme extract and added LA on CLA production, the objective of this study was to determine the effect of three levels of crude enzyme extracted from *L. acidophilus* (CCRC 14079) and added LA on CLA isomers and total CLA productions in vitro.

2. Materials and methods

2.1. Lactic culture

Lactobacillus acidophilus (CCRC 14079) was purchased from the Culture Collection and Research Center (CCRC), Food Industrial Research Institute, Shin Chu, Taiwan, and was subcultured twice under aerobic conditions at 37 °C for 24 h in MRS broth (Difco Lab., Detroit, MI)

2.2. Enzyme preparation

To prepare crude enzyme extract (1%, v/v) activated culture was inoculated into each of the four 1000 ml MRS broths and incubated toward the end of logarithmic phase at 37 °C. Following incubation, cells were harvested by centrifugation (10,000×*g* for 10 min at 4 °C) (Parra, Casal, & Gomez, 2000) washed once at 4 °C with 30 ml of 20 mM sodium phosphate buffer at pH 5, and resuspended in 30 ml buffer solution. After mixing gently with 200 mg lysozyme powder and incubating for 10 min at 20 °C (Bollag, Rozycki, & Edelstein, 1996), the washed cells were disrupted by sonication for 10 min at around 0 °C in a bath containing ice, NaCl, and ethanol. The disrupted cells were then removed by centrifugation (10,000×*g* for 60 min at 4 °C) and 480 g ammonium sulfate were slowly added to the cell-free supernatant while stirring. After standing for 1 h and centrifuging at 10,000×*g* for 30 min at 4 °C. The precipitate was dissolved in 30 ml of 20 mM pH 5 sodium phosphate buffer and dialyzed overnight at 4 °C against 2 l of buffer solution to remove ammonium sulfate (Bolton, Kelly, & Fogarty, 1997) which was monitored by FPLC equipped with a Hitrap desalting column (AKTAprime, Amersham Pharmacia Biotech Inc., Piscataway, NJ). After being filtered through 0.22 μm membrane filters, the retentate was concentrated ~3-fold by centrifugation (3000×*g* for 1 h at 4 °C) using an Amicon centrifugal filter unit fitted with a Centriplus-20 membrane of 30-kDa nominal molecular weight cutoff (Millopore Corp., Bedford, MA) for protein measurement and CLA production.

2.3. Protein measurement

Protein was measured according to Bradford's method (Bradford, 1976) with bovine serum albumin as standard.

2.4. CLA production

Each of the two levels of free linoleic acid: 50 and 75 mg was mixed with four different levels of crude LA isomerase: 0, 25, 50, and 75 mg protein. A ~15–20 ml aliquot of the mixture was then incubated at 50 °C in an orbital shaker and was shaken gently for 10 min. The reaction was terminated by the addition of an equal volume of 15% trichloroacetic acid (TCA)-5% $FeCl_3$ solution (Tsai, Lin, & Jiang, 1996).

2.5. Fatty acid extraction and preparation of fatty acid methyl esters

Following enzyme reaction, the mixture was extracted with 45 ml chloroform: methanol (2:1, v/v), and 5 mg heptadecanoic acid (Sigma Chemical Co., St. Louis, MO) were added as the internal standard for HPLC analysis. After homogenizing in a Nihon Seiki universal homogenizer (Tokyo Nihon Seiki Seisakusho Co., Tokyo, Japan) for 5 min at #4 setting, the mixture was centrifuged at 2000×*g* for 5 min at 4 °C in a refrigerated centrifuge (Himac CR20B2, Hitachi, Tokyo, Japan). The lower layer was then dried with 10 g anhydrous sodium sulfate, evaporated with a rotary evaporator at 30 °C, and flushed with nitrogen until dry (Ha & Lindsay, 1990; Ha, Grimm, & Pariza, 1989).

The residue was methylated with 14% BF_3–MeOH in a screw-capped test tube at 25 °C for 30 min (Chin et al., 1992). The methylated sample was then mixed with 2 ml hexane: water (1:1, v/v) and centrifuged at 2000×*g* for 5 min at 4 °C. Finally, the organic layer was concentrated to ~1 ml under a stream of nitrogen at room temperature for further quantification of CLA isomers by HPLC.

2.6. HPLC analysis

Instrumentation used for the analyses was as follows: A Jasco HPLC (Jasco Co., Tokyo, Japan) equipped with two ChromSpher 5 Lipids analytical silver-

impregnated columns (4.6 mm i.d.×250 mm stainless steel; 5 μm particle size; Chrompack, Bridgewater, NJ) in series (Sehat et al., 1999) a Jasco 870-UV detector operated at 233 nm, and a Jasco PU-980 pump. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 ml min^{-1} (Sehat et al., 1999). The column head pressure was maintained at 48 atm at this flow rate. Whenever necessary, the column was restored by flushing with 1% acetonitrile in hexane for 2–4 h, followed by 1–2 h with 0.1% acetonitrile in hexane. A Rheodyne 7725i injector (Rheodyne, L.P. Cotati, CA) with 50 μl injection loop was used and the injection volume was 10 μl. The results were analyzed by a SIC Chromatocoder12 integrator (System Instrument Co., Ltd., Tokyo, Japan).

Eight CLA methyl esters, eluted between 15 and 30 min, were identified by comparing the retention times with the methylated CLA standard (Sigma Chemical Co., St. Louis, MO). The peak of t8,t10-CLA was first eluted followed by t9,t11-, t10,t12-, t11,t13-, t8,c10-, c9,t11-, t10,c12c-, and c11,t13-CLA. After computing the amounts of all eight standard CLA isomers using area %, the areas of the sample peaks were further calculated as μg CLA, using heptadecanoic acid as the internal standard. Total CLA was obtained by summing the levels of those isomers calculated. Level of each isomer was divided by total CLA level and was expressed as% CLA isomer in total CLA.

2.7. Statistical analysis

Enzyme extraction was performed in three replications and six CLA-producing reactions were done in each replication. The data obtained from those replications were subjected to 2-way MANOVA and Duncan's multiple range test and critical ranges using STATISTICA (StatSoft, 1998) and a significance level of 0.05 was used.

3. Results and discussion

Eight CLA isomers: t8,t10-, t9,t11-, t10,t12-, t11,t13-, t8,c10-, c9,t11-, t10,c12c-, and c11,t13-CLA, were detected in the enzyme reactions with LA addition, as shown in Table 1, and the retention times of trans,trans- and *cis*,*trans*/*trans*,*cis*-CLA in HPLC chromatogram were 17–20 and 22–25 min, respectively.

The sharp increases ($P < 0.05$) in total CLA levels from 8±1 to 305±17 μg in 50 mg LA treatment and from 116±2 to 439±23 μg in 75 mg LA treatment were observed as enzyme extract level increased from 0 to 50 mg (Fig. 1), which demonstrated the presence of LA isomerase activity in the crude enzyme extract. Because 75 mg LA reacted with more enzyme active sites, higher total CLA levels were produced in the 75 mg LA treatment, than with 50 mg LA ($P < 0.05$). However, since all the added LA may have fully reacted with the active sites of 50 mg enzyme, total CLA level remained unchanged ($P > 0.05$) as enzyme level increased to 75 mg. CLA was detected in the controls of 50 and 75 mg LA/0 mg enzyme, possibly due to the presence of small amounts of CLA in the added LA and the occurrence of LA conjugation during incubation (Ha et al., 1989; Shantha, Decker, & Ustanol, 1992). Higher total CLA level in the 75 mg LA/0 mg enzyme treatment than in 50 mg LA/0 mg enzyme treatment probably also resulted from a higher CLA level and LA conjugation rate in the 75 mg LA.

The distributions of some CLA isomers were different between the controls and the enzyme-added treatments (Table 1). The significant decreases in t11,t13-CLA% from 40–45 to 4–28% were observed ($P < 0.05$) in all the enzyme treatments as compared with the controls, whereas, significant increases in t9,t11-CLA%, from 3–6 to 10–44% were observed ($P < 0.05$) in four enzyme treatments. The significant increases in isomer levels were also observed ($P < 0.05$) in certain enzyme treatments of t10,t12-, t8,c10-, c9,t11-, and c11,t13-CLA. The shifts in

Table 1
Percentages of CLA isomers in total CLA produced in the reactions of enzyme extract and LA

Enzyme extract (mg)	LA (mg)	CLA isomer (%)							
		Trans,*trans*-CLA				*Trans*,*cis*/*cis*,*trans*-CLA			
		t8,t10	t9,t11	t10,t12	t11,t13	t8,c10	c9,t11	t10,c12	c11,t13
0	50	0a	6a	6a	45a	8a	19a	5a	11a
	75	4bc	3a	10ab	40a	2b	5b	25ab	11a
25	50	7c	28b	7a	20b	11c	12abc	3a	12a
	75	4bc	24b	24c	17bc	5d	11bc	8ab	7ab
50	50	3ab	4a	20bc	21b	0e	13ac	36b	4b
	75	6bc	10c	9a	4c	5d	32d	22ab	11a
75	50	8c	44d	4a	2c	2b	14ac	17ab	8ab
	75	8c	3a	22c	28b	24e	4b	4a	24c
Average[a]		6	19	14	15	8	14	15	11

Means of each CLA isomer % in the same column followed by the same letters are not significantly different ($P > 0.05$).
[a] Average of CLA isomer % in six enzyme treatments.

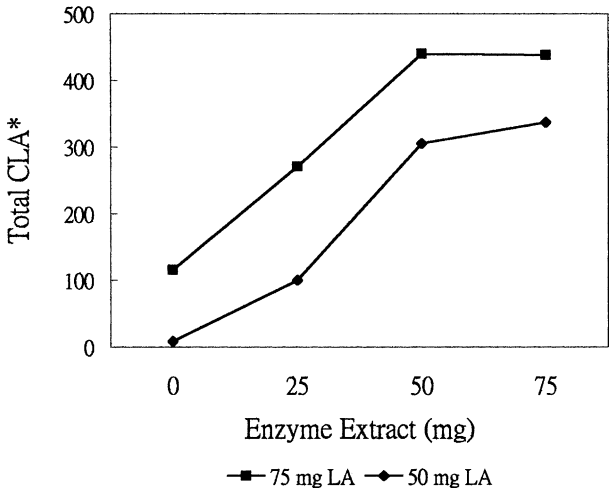

Fig. 1. Levels of total CLA (μg) produced in the reactions of enzyme extract and LA. *Relative standard deviations (RSDs) for the treatments of 75 and 50 mg LAs were 0.9–5.3 and 0.1–5.9%, respectively.

distributions of those CLA isomers could be due to the enzymatic intraisomerization (Kramer et al., 1997; Shantha, Decker, & Henning, 1993; Werner, Luedecke, & Shultz, 1992), which converted t11,t13-CLA into other CLA isomers during incubation. The distribution of CLA isomer produced varied with enzyme and LA additions. The largest differences ranging from 3 to 44%, were observed in t9,t11-CLA, whereas the smallest differences ranging from 3 to 8%, were observed in t8,t10-CLA. Different isomer distribution in added LA of various replications and presence of impurities in the enzyme extract, e.g. proteases (Bollag et al., 1996) or cation (Kreft & Jelen, 2000), which may affect the enzyme activity (Price & Stevens, 1989) to a certain degree, were two possible reasons for the difference in isomer distribution in the enzyme treatments.

The average CLA isomer % in six enzyme treatments ranged from 6 to 19% (Table 1). Forty-eight percent of those isomers were in *cis,trans*/*trans,cis* form, lower than the 80% in the diet supplements reported by Christie, Dobson, and Gubstone (1997). The percentage of c9,t11-CLA, the major biologically active isomer (Ha & Lindsay, 1990; Ip et al., 1991; Pariza et al., 2001), was 14% in the products of the enzyme catalyzed reaction, which was also lower than the 75% reported for diet supplements (Chin et al., 1992). Nevertheless, the CLA isomers were distributed more evenly in the enzyme reactions, and the average percentages of *cis,trans*/*trans,cis*-CLAs: t8,c10-, c9,t11-, t10,c12-, and c11,t13-CLA were 8, 14, 15, and 11%, respectively.

4. Conclusion

The sharp increases in total CLA levels, from 8 to 305 μg in the 50 mg LA treatment and from 116 to 439 μg in the 75 mg LA treatment, were observed as enzyme extract level increased from 0 to 50 mg, which demonstrated the presence of LA isomerase activity in the crude enzyme extract of *Lactobacillus acidophilus* (CCRC 14079) and the feasibility of CLA production through the enzyme method. Forty-eight percent of CLA isomers in total CLA produced were in cis,trans/trans,cis form and 14% were c9,t11-CLA, lower than those reported in the diet supplements, indicating more even distribution of CLA isomers produced through the enzyme reaction. Further research is needed to study LA isomerase kinetics using more enzyme levels and further purified enzyme.

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