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Conjugated linoleic acid production by cells and enzyme extract of *Lactobacillus delbrueckii* ssp. *bulgaricus* with additions of different fatty acids

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Abstract

The objective of the study was to determine the effect of fatty acid additions to the cells and enzyme extract of *Lactobacillus delbrueckii* ssp. *bulgaricus* (CCRC14009) on CLA production. Washed cells of *L. delbrueckii* ssp. *bulgaricus*, obtained by cultivation in a MRS broth, were mixed with BSA and each of the three fatty acids: linoleic, oleic, and linolenic acids in sodium phosphate buffer at pH 6.5. After incubation at 37 °C for 108 h, CLA concentration was analyzed by HPLC. Enzyme extract from the culture was also reacted with each fatty acid at 50 °C for 10 min at pH 5 to test for CLA production. Results showed that linoleic acid addition to the culture improved CLA production, indicating the presence of linoleic acid isomerase activity in the culture. The crude enzyme extract from the culture was observed to be capable of oleic and linolenic acid conversions into CLA, demonstrating the possible presence of desaturase activity in the enzyme extract.

Keywords: *Lactobacillus delbrueckii* ssp. *bulgaricus*; Conjugated linoleic acid; Linoleic acid; Oleic acid; Linolenic acid

1. Introduction

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of octadecadienoic fatty acid with conjugated double bonds. These conjugated dienes are attracting interest because of their potential health benefits in the area of cancer (Belury, 2002), atherosclerosis (Wilson, Nicolosi, Chrysam, & Kritchevsky, 2000), diabetes (Houseknecht et al., 1998), obesity (Delany, Blohm, Truett, Scimeca, & West, 1999; Rainer & Heiss, 2004), and immune function (Hayek et al., 1999).

CLA occurs naturally in a variety of foods, including meat, poultry, seafood, cheese, butter, milk and vegetable oils (Ip, Chin, Scimeca, & Pariza, 1991). Ruminant fats are the richest natural sources of CLA among those products (Chin, Liu, Storkson, Ha, & Pariza, 1992; Shantha, Crum, & Decker, 1994). The high CLA levels in ruminant depot fat originate partly from ruminal bacteria (Shorland, Weenink, & Johns, 1995) due to the presence of linoleic acid (LA) isomerase, converting linoleic acid into CLA in the rumen (Chin, Storkson, Liu, Albright, & Pariza, 1994; Yang & Pariza, 1995) and Δ^9-desaturase converting *trans*-vaccenic acid (C18:1 *trans*-Δ^{11}) into c9,t11-CLA in the intramuscular fat (Raes, De Smet, & Demeyer, 2004). The presence of Δ^9-desaturase, activity was also found in the mammary gland (Corl et al., 2001; Griinari et al., 2000), in which CLA synthesis was estimated to account for 78% of the total c9,t11-CLA in milk fat (Sieber, Collomb, Aeschlimann, Jelen, & Eyer, 2004). The presence of LA isomerase activity was also observed in several strains of

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propionibacteria (Jiang, Bjröck, & Fondén, 1998) and a lactic acid bacterium (Lin, Lin, & Wang, 2002).

A plant Δ^{12}-oleic-acid desaturase-related enzyme that modified a Δ^{9}-double bond to produce the conjugated *trans*-Δ^{8}, *trans*-Δ^{10}-double bonds found in calendic acid (C18:3 *trans*-Δ^{8}, *trans*-Δ^{10}, *cis*-Δ^{12}) was reported by Cahoon, Ripp, Hall, and Kinney (2001). Decreases in CLA contents of three cheddar-type cheeses during additional aging were observed, possibly due to desaturase or elongase action (Lin, Boylston, Luedecke, & Hultz, 1999). Furthermore, an endogenous synthesis of c9,t11-CLA from *trans*-vaccenic acid, through desaturation, was shown in mice (Santora, Palmquist, & Roehrig, 2000). However, no research regarding the presence of desaturase activity in lactic acid bacteria has been done. We recently demonstrated the presence of LA isomerase activity in the enzyme extract of *Lactobacillus acidophilus* CCRC14079 (Lin, Lin, & Wang, 2003). In order to examine the presence of LA isomerase and desaturase activities in *L. delbrueckii* ssp. *bulgaricus* (CCRC14009), this study was aimed at determining the effects of oleic, linolenic, and linoleic acid additions to the cells of *L. delbrueckii* ssp. *bulgaricus* (CCRC14009), and the enzyme extract from the cells, on CLA production.

2. Materials and methods

2.1. Culture of cells

Lactobacillus delbrueckii ssp. *bulgaricus* (CCRC-14009), obtained from Culture Collection and Research Center (CCRC), Food Industrial Research Institute, Shin Chu, Taiwan were subcultured twice under aerobic conditions at 37 °C for 24 h in MRS broth (Difco Lab., Detroit, MI, USA). One percent of the subcultures was then inoculated into 1000 ml MRS broth (v/v) and incubated toward the end of the logarithmic phase at 37 °C.

2.2. Preparation of cell culture for CLA productions

Following incubation, cells of *L. delbrueckii* ssp. *bulgaricus* were harvested by centrifugation (10,000*g* for 10 min at 4 °C) (Parra, Casal, & Gomez, 2000) and washed twice with 30 ml of 0.85% sodium chloride at 4 °C. After centrifugation again, an aliquot of 1.15 g of the cells were mixed with 0.065 g BSA and 0.13 g of each of the three fatty acids: linoleic acid, oleic acid, and linolenic acid in 3.655 ml of 20 mM sodium phosphate buffer at pH 6.5 (Kishino, Ogawa, Omura, Matsumura, & Shimizu, 2002), making up the total cell count to 1.28×10^{10} CFU/ml, determined by plating on MRS agar. After incubation at 37 °C for 108 h in an orbital shaker at speed 3 (Heidolph Titramax 1000, Germany), fatty acids were extracted from the mixture and methylated for CLA analysis.

2.3. Enzyme preparation

To prepare crude enzyme extract, cells after incubation were harvested by centrifugation (10,000*g* for 10 min at 4 °C) (Parra et al., 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, USA). After being filtered through 0.22 μm membrane filter, 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 (Millipore Corp., Bedford, MA, USA) for protein measurement and CLA productions.

2.4. Protein measurement

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

2.5. CLA productions

An aliquot of 25 mg of each of the three fatty acid, linoleic acid, oleic acid, and linolenic acid, was mixed with crude LA isomerase of 50 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).

2.6. Extraction and methylation for CLA analyses

Following incubation, total volume of each of the three fatty acid mixtures was extracted with chloroform: methanol (2:1, v/v) and methylated with 14% BF_3–MeOH according to the method of Lin et al. (2002).

The methylated sample was mixed with 2 ml hexane:water (1:1, v/v), centrifuged at 2000*g* for 5 min at 4 °C, and the organic layer was concentrated to ~1 ml under a stream of nitrogen at room temperature for further quantification of CLA isomers by HPLC.

2.7. CLA quantification by HPLC

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, USA) 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, USA) with 50 μl injection loop was used and the injection volume was 10 μl. The results were analyzed by a SISC32 Chromatography Data Station (SISC, Taipei, Taiwan).

Ten to thirteen 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, USA). After computing the amounts of all the 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 up the levels of those isomers calculated.

2.8. Statistical analysis

Each fatty acid addition for CLA production was performed in three replications and three CLA analyses were done for each replication. The data obtained from those replications were subjected to general 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

A significant increase in total CLA level from 27.0 to 209 μg was observed ($P < 0.05$) in the reaction of washed cells of *L. delbrueckii* ssp. *bulgaricus* with added linoleic acid (LA) (Table 1), indicating the presence of the activity of LA isomerase, converting LA into CLA in the cells. The result was in accordance with those obtained by Kishino et al. (2002), who reported CLA production from the free form of LA by washed cells of 14 genera of lactic acid bacteria excluding *L. delbrueckii* ssp. *bulgaricus*. Since the amounts of t,t-, c,t/t,c-, and c,c-CLA isomers produced were 56.0, 121, and 32.1 μg, respectively, in the LA treatment, which were significantly higher ($P < 0.05$) than those of the control and two other fatty acid treatments, LA addition to the washed cells of *L. delbrueckii* ssp. *bulgaricus* appeared to be effective in promoting the production of those three groups of CLA isomers. In addition, the yields of t10,c12-, t10,t12-, and c9,t11-CLA were larger than those of other isomers and were the major CLA isomers produced in LA treatment. Neither oleic acid nor linolenic acid addition showed any significant increase in total CLA level ($P > 0.05$) compared to the control, indicating ineffectiveness in enhancing total CLA production by the washed cells with the additions of those two fatty acids.

A significant increase in total CLA production, from 0.1 to 8.5 μg, was observed ($P < 0.05$) as LA was reacted with the crude enzyme extract of *L. delbrueckii* ssp. *bulgaricus* cells (Table 2), which further demonstrated the presence of LA isomerase activity in the enzyme extract of the cells. The result was in accordance with results obtained by Lin et al. (2002), who reported the presence of LA isomerase activity in *L. acidophilus*. However, the yields of each CLA isomer and total CLA produced by the enzyme extract in LA treatment were sharply lower than those produced by the washed cells (8.5 vs. 209 μg in total CLA). The difference in reaction

Table 1
CLA production by washed cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* with additions of linoleic, linolenic and oleic acids

CLA	CLA level (μg)			
	Linoleic acid	Linolenic acid	Oleic acid	Control
t12,t14-	0.5^{x}	0.1^{x}	1.2^{x}	ND**
t11,t13-	3.7^{xy}	2.1^{y}	8.9^{x}	0.8^{y}
t10,t12-	31.6^{x}	7.4^{y}	11.3^{y}	4.7^{y}
0t9,t11-	16.6^{x}	3.3^{y}	9.8^{xy}	2.3^{y}
0t8,t10-	3.6^{x}	2.1^{x}	5.1^{x}	1.7^{x}
c11,t13-	19.4^{x}	2.8^{y}	1.4^{y}	0.7^{y}
t10,c12-	57.2^{x}	7.3^{y}	14.1^{y}	8.2^{y}
0c9,t11-	30.9^{x}	5.5^{y}	10.4^{y}	2.2^{y}
0t8,c10-	13.0^{x}	1.9^{y}	1.4^{y}	0.9^{y}
c11,c13-	7.3^{x}	2.3^{y}	0.8^{y}	1.6^{y}
c10,c12-	13.4^{x}	2.3^{y}	3.6^{y}	1.9^{y}
0c9,c11-	6.3^{x}	1.8^{y}	0.1^{y}	1.4^{y}
0c8,c10-	5.1^{x}	1.0^{y}	1.1^{y}	0.6^{y}
t,t-	$56.0^{x,a}$	$15.0^{y,a}$	$36.3^{y,a}$	$9.5^{y,a}$
c,t-/t,c-	$121^{x,b}$	$17.5^{y,a}$	$27.3^{y,b}$	$12.0^{y,a}$
c,c-	$32.1^{x,c}$	$7.3^{y,a}$	$5.6^{y,c}$	$5.5^{y,a}$
Total	209^{x}	39.8^{y}	69.2^{y}	27.0^{y}

[xy] Means in the same row followed by the same superscripts are not significantly different ($P > 0.05$).
[abc] Means in the same column followed by the same superscripts are not significantly different ($P > 0.05$).
** Not detected.

Table 2
CLA production by enzyme extract of *Lactobacillus delbrueckii* ssp. *bulgaricus* cells with additions of linoleic, linolenic and oleic acids

CLA	CLA level (μg)			
	Linoleic acid	Linolenic acid	Oleic acid	Control
t12,t14-	ND**	1.3	ND	ND
t11,t13-	ND	0.2	ND	ND
t10,t12-	ND	0.2	ND	ND
0t9,t11-	0.1^{x}	0.2^{x}	0.5^{x}	0.1^{x}
0t8,t10-	0.8^{x}	1.6^{x}	1.3^{x}	ND
c11,t13-	1.4	ND	ND	ND
t10,c12-	0.9^{x}	ND	0.6^{x}	ND
0c9,t11-	3.1^{x}	ND	1.1^{y}	ND
0t8,c10-	2.2	ND	ND	ND
0t7,c9-	ND	ND	0.4	ND
t,t-	$0.9^{xz,a}$	3.5^{y}	$1.8^{x,a}$	0.1^{z}
c,t-/t,c-	$7.6^{x,b}$	ND	$2.9^{y,a}$	ND
c,c-	ND	ND	ND	ND
Total	8.5^{x}	3.5^{y}	4.7^{y}	0.1^{z}

[xyz] Means in the same row followed by the same superscripts are not significantly different ($P > 0.05$).
[ab] Means in the same column followed by the same superscripts are not significantly different ($P > 0.05$).
** Not detected.

condition between the washed cells and enzyme extract studies probably resulted in the large variation in CLA yield. Another reason might be lower enzyme activity in the crude extract, due to the complicated and long enzyme extraction and purification procedures. Since Lin et al. (2003) reported 305 μg of total CLA produced by the enzyme extract of *L. acidophilus* with 50 mg LA addition, which was higher than the amounts produced by the cells and the enzyme extract with 25 mg LA addition in this study, the LA isomerase activity in *L. delbrueckii* ssp. *bulgaricus* appeared to be lower than in *L. acidophilus*. Nevertheless, because higher level of LA addition could also affect the CLA yield, increase in LA addition, to 50 mg or higher, needs to be tested for enhancing CLA production by the enzyme extract of *L. delbrueckii* ssp. *bulgaricus*.

The total CLA level was higher in the oleic acid treatment than in the control (Table 2), demonstrating the presence of enzymes in the crude enzyme extract capable of oleic acid conversion into CLA. Since fatty acid desaturase-related enzymes were responsible for converting oleic acid into *trans*-linoleic acid in dimorphecolic acid (C18:2 *trans*-Δ^{10}, *trans*-Δ^{12}) production (Cahoon et al., 2003) and modifying a Δ^{9} double bond into the conjugated *trans*-Δ^{8}, *trans*-Δ^{10}-double bonds in calendic acid production (Cahoon et al., 2001), the enzymes in the crude enzyme extract could include desaturase which either desaturated added oleic acid into linoleic acid for further CLA formation by LA isomerase or directly converted oleic acid into $\Delta^{8,10}$-CLA, by modifying the Δ^{9} double bond. Higher total CLA level was also observed in linolenic acid treatment (Table 2), indicating the presence of enzymes in the crude enzyme extract capable of linolenic acid conversion into CLA. Because Harfoot and Hazlewood (1988) observed *trans*-vaccenic acid as one of the final products of linolenic acid biohydrogenation but did not find any CLA as an intermediary agent, those enzymes in the crude enzyme extract could include enzymes of biohydrogenation, catalyzing the formation of *trans*-vaccenic acid and Δ^{9}-desaturase and further converting *trans*-vaccenic acid into $\Delta^{9,11}$-CLA (Corl et al., 2001; Griinari et al., 2000; Raes et al., 2004).

Although the enzyme extract of *L. delbrueckii* ssp. *bulgaricus* cells was found to be capable of CLA production with oleic and linolenic acid additions, no significant increase in total CLA production by the washed cells of *L. delbrueckii* ssp. *bulgaricus* was observed ($P > 0.05$) as oleic or linolenic acid was added, possibly due to the different reaction conditions. The condition of pH 6.5, 37 °C and 108 h reaction time, used in the washed cells study, favoured LA conversion into CLA but disfavoured oleic and linolenic acid conversions, whereas the condition of pH 5, 50 °C and 10 min reaction time, used in the enzyme extract study, favoured all three fatty acid conversions into CLA but with low yield.

4. Conclusions

Linoleic acid addition, to either the washed cells of *L. delbrueckii* ssp. *bulgaricus* or the crude enzyme extract, improved CLA production, indicating the presence of linoleic acid isomerase activity in the culture. The enzyme extract from the cells was observed to be capable of oleic and linolenic acid conversions into CLA, demonstrating the possibility of desaturase activity in the enzyme extract. This is the first report of desaturase activity in lactic acid bacteria. However, the enzyme activity was low and further investigation on improving desaturase activity, including optimizing the enzyme extraction and CLA production conditions is needed.

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