African Journal of Biotechnology Vol. 11(20), pp. 4579-4587, 8 March, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3632
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Purification and characterization of a linoleate isomerase from *Lactobacillus plantarum* ZS2058

Haiqin Chen[#], Bo Yang[#], Sitian Gu, Baixi Zhang, Qingyan Xu, Qiang Ye, Yuanda Song, Yong Q. Chen, Hao Zhang and Wei Chen*

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, People's Republic of China.

Accepted 17 February, 2012

Linoleate isomerase (EC 5.2.1.5) catalyzes the isomerization of linoleic acid to generate conjugated linoleic acid. Previously, we isolated a strain of *Lactobacillus plantarum* ZS2058 with great capacity for producing conjugated linoleic acid from fermented vegetables. This work aimed to purify the linoleate isomerase from *L. plantarum* ZS2058 and investigate its characteristics. Electrophoresis of the purified enzyme by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed a single band of protein with a molecular mass of 66 kDa. The purified linoleate isomerase was active, with a specific activity of 3.71 nmol/ [min· (mg·protein)] and a K_m of 21.5 µM for linoleic acid. The optimal pH and temperature for enzyme activity were determined to be pH 6.5 and 35°C respectively. No external cofactors or energy sources were required for this activity. Metal chelators, ethylenediamine tetra-acetic acid (EDTA) and ethylene glycol tetraacetic acid and (EGTA), and metal ions had no effect on enzyme activity.

Key words: Conjugated linoleic acid, *Lactobacillus plantarum* ZS2058, linoleate isomerase, purification, characterization.

INTRODUCTION

Conjugated linoleic acids (CLAs) are a family of at least 28 isomers of linoleic acid (LA), found rarely in meat and dairy products derived from ruminants (Banni, 2002). Biological activities associated with the cis-9, trans-11 CLA (c9, t11 CLA) and the trans-10, cis-12 CLA (t10, c12 CLA) isomers include anti-cancer, anti-atherosclerotic, anti-diabetic and immune-enhancing properties. In addition, they have positive effects on body composition and bone formation (Banni et al., 2003; Belury, 2002, 2003; Kritchevsky et al., 2004; Pariza et al., 1999, 2000). The c9, t11 and t10, c12 CLA isomers are recognized as the most biologically active CLA species. Although methods have been described for the isolation of CLAs from chemically synthesized mixtures, these processes are expensive and often do not provide high purities of single isomers (Haas et al., 1999; Mounts et al., 1970). Potentially, the use of biological processes for producing CLAs may solve these problems.

Dairy products are the major natural sources of CLAs, while c9, t11 CLA is the most commonly found isomer (Chin et al., 1992). CLAs in dairy products are produced from polyunsaturated fatty acids by certain rumen microorganisms (Griinari and Bauman, 1999). The c9, t11 CLA may be an intermediate in the biohydrogenation of LA to stearic acid by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens* (Kepler et al., 1966). Additional studies have shown that other bacteria can transform LA into CLA (mainly the c9, t11, t10 and c12 isomers), including *Propionibacterium freudenreichii*, *Streptococcus salivarius*, *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Clostridium sporogenes, Clostridium bifermentans, Clostridium*

*Corresponding author. E-mail: weichen@jiangnan.edu.cn. Tel/Fax: +86-510-85912155.

Abbreviations: CLA, Conjugated linoleic acid; **PPB,** potassium phosphate buffer; **LA,** linoleic acid.

# Both authors contributed equally to this work.

sordellii and *Bacteroides* spp. (Alonso et al., 2003; Lee et al., 2003; Lin et al., 1999; Ogawa et al., 2001, 2005; Rainio et al., 2002; Verhulst et al., 1985). Linoleate isomerase, which acts on the C9 and C12 double bond has been purified from *P. freudenreichii*, *Propionibacterium acidipropionici*, *Propionibacterium acnes*, *L. acidophilus*, *L. reuteri*, *C. sporogenes* and *B. fibrisolvens* (Kepler and Tove, 1967; Lin et al., 2002; Peng et al., 2007).

Developing processes for the production of single isomers of CLA using microbial cells and enzymes is of significant commercial interest (Ando et al., 2004; Kim et al., 2000; Rainio et al., 2001). To realize this potential, the first step towards identifying linoleate isomerase genes is to purify the isomerases themselves. In our previous study, *L. plantarum* ZS2058 isolated from Chinese traditional fermented vegetables, showed a great capacity to convert LA into CLA, with conversion efficiencies of greater than 40%. The main products of the bioconversion of LA by the resting cells of *L. plantarum* ZS2058 were the c9, t11; t10, c12; and t, t isomers of CLA. Bioactive isomers of CLA constituted more than 50% of the products. Hence, *L. plantarum* ZS2058 is potentially useful for producing CLAs on a large scale. This study therefore describes the purification and characterization of the linoleate isomerase from *L. plantarum* ZS2058, with the aim to investigate its enzymatic efficiency.

MATERIALS AND METHODS

Cultures, media and growth conditions

L. plantarum ZS2058 previously isolated from Chinese traditional fermented vegetables in our laboratory was provided by the Culture Center of Food Microorganisms of Jiangnan University (CCFM-JU, Wuxi, China). This strain was subcultured three times in de Man, Rogosa and Sharpe (MRS) culture medium supplemented with 1% Tween-80 and 1 mg/ml LA for 12 h at 37°C.

Fatty acid analysis by gas chromatography

Lipid extraction and preparation of fatty acid methyl esters (FAMEs) were performed according to the procedures of Bligh and Dyer (1959). FAMEs were extracted with n-hexane and analyzed by gas chromatography (GC) using a GC-2010 gas chromatograph equipped with a flame ionization detector (Shimadzu; Kyoto, Japan). Injections of 1 µL were performed automatically using a split ratio of 1:5. Hydrogen and helium were used as the carrier gases through a BPX-70 column (120 m × 0.25 mm; i.d. 0.25 µM; SGE, France). The column temperature was initially at 150°C before it was increased to 190°C at a rate of 10°C/min, and was kept at this temperature for 0.1 min. Then the temperature was increased to 220°C at a rate of 2°C/min and this was maintained for 15 min. The injector and detector were operated at 250°C.

Linoleate isomerase extraction

L. plantarum ZS2058 cultures were harvested by centrifugation at 7000 rpm for 20 min. Cells were washed three times with potassium phosphate buffer (PPB; 0.1 M, pH 6.5), and resuspended in fresh PPB to a cell concentration of 70 mg/ml. The suspension was digested with 50 mg/ml lysozyme and ultrasonicated. Then the cell lysate was extracted with 1% Triton X-100 at 4°C for 12 h, and the cell free extract was prepared by centrifuging at 4°C and 9000 rpm for 30 min. The supernatant was kept and designated the crude extract.

Linoleate isomerase activity assay

The reaction mixture contained 2 ml extract and a filtered-sterilized emulsion of LA at a final concentration of 0.08 mg/ml. The emulsion composed of LA and Tween-80 (5:1) in water that was exposed to ultrasonic emulsification for 1 min. The concentration of LA in this emulsion was 50 mg/ml. Reactions were carried out in a 10 ml screw-cap tube at 37°C for 12 h with shaking (200 stroke/min) and stopped by adding 10% trichloroacetic acid. Subsequently, 4 ml n-hexane was added into the reaction mixture, followed by shaking (200 stroke/min) for 1 h for extracting the fatty acid, then centrifuging at 6000 rpm at 4°C for 10 min. And finally, the absorption of organic phase was determined at 234 nm. The absorbance at 234 nm (A_{234}), characteristic of conjugated double bonds in fatty acids (Kepler and Tove, 1967) was also measured to quantify the CLAs. If there was Triton X-100 in the reaction system, 0.5 ml methanol and 0.5 ml 5 M NaCl solution were added before extraction to improve the isolation of organic phase from water phase. Meanwhile, the control reactions (without isomerase) did not produce detectable amounts of CLAs. An active unit of linoleate isomerase was defined as the amount of enzyme that was required to produce 1 µg CLAs per hour.

Linoleate isomerase purification

Ammonium sulfate fractionation

Ammonium sulfate was added to the crude extract at different saturations (10 to 90%) for obtaining the optimal range. After each addition, the crude extract was precipitated for 1 h at 0°C and centrifuged for 1 h at 10,000 × g and 4°C, and the isomerase activity and protein concentration in supernatant was assayed. With the optimal range, the supernatant was precipitated with ammonium sulfate at the lowest limit and re-precipitated under the same conditions as before. Finally, the precipitate was re-dissolved in PPB (0.1 M, pH 6.5, with 5% glycerol), and then the supernatant was treated at the upper limit.

Desalting and concentrating

The final suspension after ammonium sulfate fractionation was put into a dialysis tube (MWCO 2000; Millipore, US) and kept overnight in Tris-HCl buffer (20 mM, pH 7.5, with 1% Triton X-100 and 5% glycerol) at 4°C. The desalted suspension was concentrated by ultrafiltration (MWCO 5000; Millipore, US), and centrifuged at 8,000 × g and 4°C to the desired final volume.

Ion-exchange chromatography on diethylaminoethyl cellulose (DEAE)

The dialyzed protein samples were applied to a DEAE-Sepharose F.F column (Φ1.6 × 10 cm; Pharmacia, US), pre-equilibrated with Tris-HCl buffer (20 mM, pH 7.5, with 1% Triton X-100 and 5% glycerol). The column was washed at 2.0 ml/min using a gradient of Tris-HCl buffer lacking NaCl, and the protein was eluted by the same buffer containing 0.75 M NaCl. Fractions of 5 ml were collected, desalted and then concentrated by ultrafiltration.

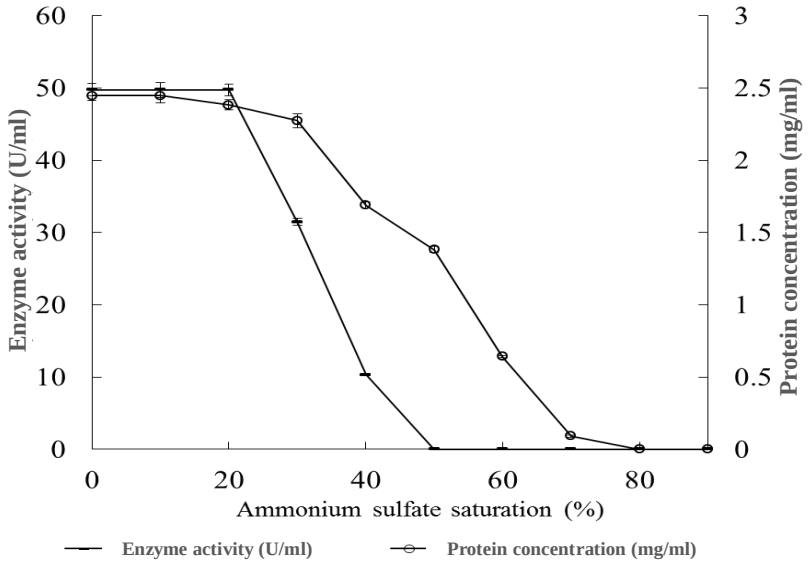

Figure 1. The effect of ammonium sulfate saturation on assaying the enzyme activity and protein concentration in the supernatant.

Gel filtration chromatography

Fractions from the DEAE-Sepharose F.F column were applied to a Sephadex G-100 column (Φ1.0 × 100 cm; Pharmacia, US) equilibrated with PPB (0.1 mM, pH 6.5, with 1% Triton X-100 and 5% glycerol). Protein elution was performed at 0.3 ml/min using an AKTA-Primer Basic System (GE Healthcare, Sweden). Fractions of 3 ml were collected and those corresponding to linoleate isomerase were combined and concentrated.

Protein analysis

The quantity of protein collected was assayed according to the Peterson method (Peterson, 1983) using bovine serum albumin as the standard. To determine their molecular weight, samples were separated by 12% Tris-Glycine SDS-PAGE.

Assays for enzymatic characterization and kinetic studies

Optimization of temperature and pH

The optimum temperature and pH of the linoleate isomerase were determined by measuring enzyme activity at various temperatures (30, 35, 40 and 45°C; each at pH 6.5) and pH (4.5, 5.5, 6.5, 7.5 and 8.5; each at 37°C) in PPB using LA as the substrate.

Thermostability of the enzyme

To determine the thermostability of the enzyme, the sample was incubated at 30, 50 and 70°C, and the enzyme activity was assayed every 2 h for up to 10 h.

Effect of various metal ions and metal chelators

Enzyme activity assays were performed in the presence of various metal ions (Mg^{2+}, Ca^{2+}, Al^{3+}, Cu^{2+}, Ba^{2+}, Zn^{2+}, Pb^{2+}, Fe^{2+}, Fe^{3+} and Mn^{2+}) and metal chelators (ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)) at final concentrations of 1 mM. Each reaction was incubated for 3 h at pH 6.5 and 37°C.

Effect of cofactors

Certain cofactors (ATP, ADP, NAD, NADH and NADPH) at a final concentration of 50 µM were used to assess their effects on enzyme activity. Reactions were incubated for 3 h at pH 6.5 and 37°C.

Determination of K_m and V_{max} Value

The K_m and V_{max} values of the enzyme were determined by measuring reaction velocities at four concentrations of substrate (LA), ranging from 0.01 to 0.04 mg/ml. At 3 h, CLA concentration was determined by measuring at A_{234}. The reciprocal of the reaction velocity (1/V) was plotted against the reciprocal of the substrate concentration (1/[S]) to determine K_m and V_{max} values by the Line-Weaver-Burke plot.

RESULTS AND DISCUSSION

Purification of linoleate isomerase, enzyme assay and protein assay

The crude enzyme was purified by ammonium sulfate precipitation. Figure 1 shows that the optimal range of saturation of ammonium sulfate was 20 to 50%, as this gave acceptable activity and concentration values. The *L. plantarum* ZS2058 linoleate isomerase was purified by DEAE-Sepharose F.F (Figure 2A to D) and gel filtration chromatography (Figure 2E). Figure 2A to D showed that a high peak was obtained at pH 9.0, but not for pH 7.5,

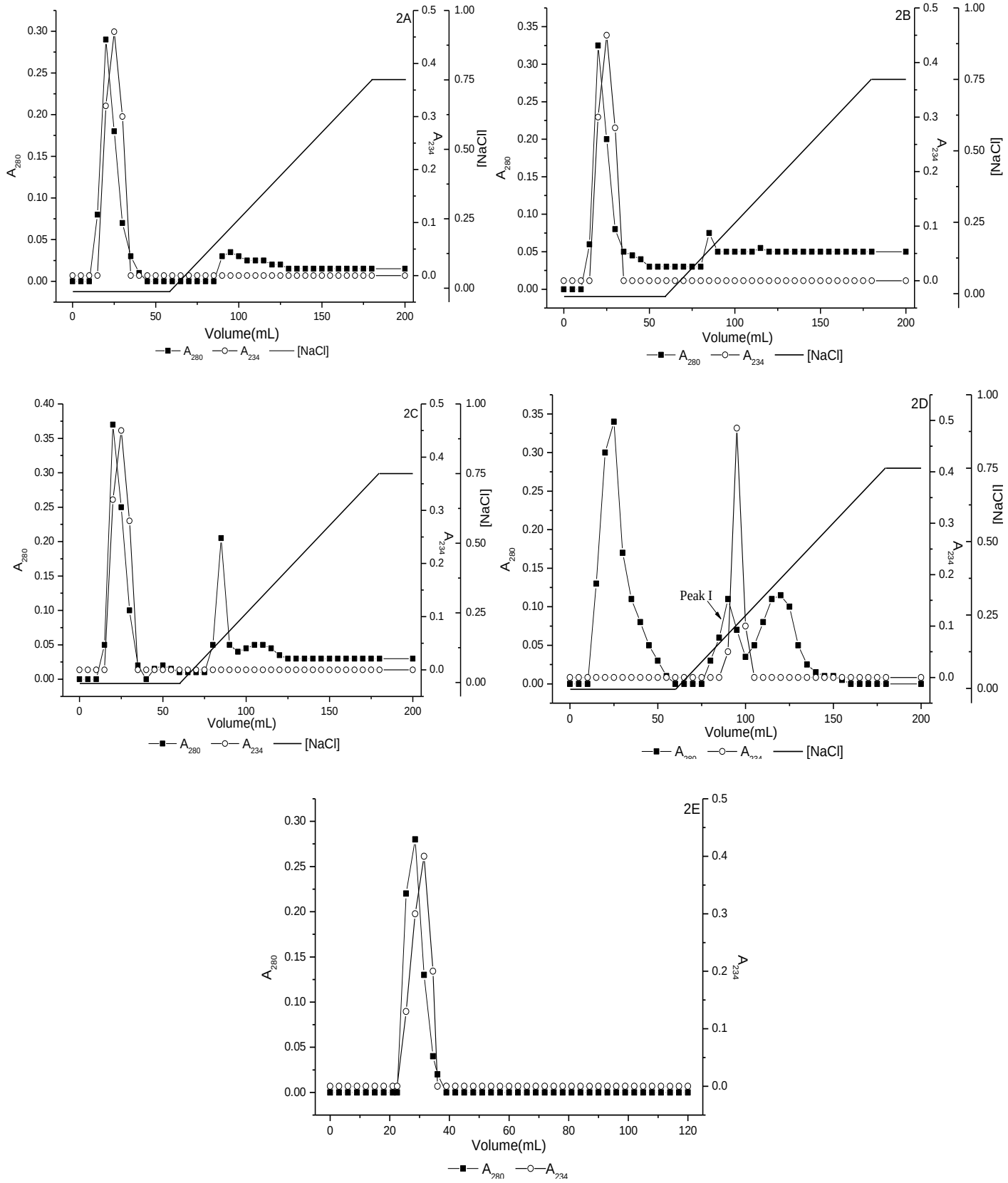

Figure 2. Purification of *L. plantarum* ZS2058 linoleate isomerase. A to D are the chromatography of DEAE-Sepharose F.F column with different pH and NaCl gradient. (A) pH 7.5, no peak was shown; (B) pH 8.0, no good isolated peak was revealed; (C) pH 8.5, no isomerase activity peak was shown; (D) pH 9.0, two peaks were shown, but only the peak I was active. The column was eluted with a of NaCl gradient 0 to 0.75 M. (E) The chromatography of Sephadex G-100 gel filtration (PPB with 1% Triton X-100 and 5% glycerol as elution).

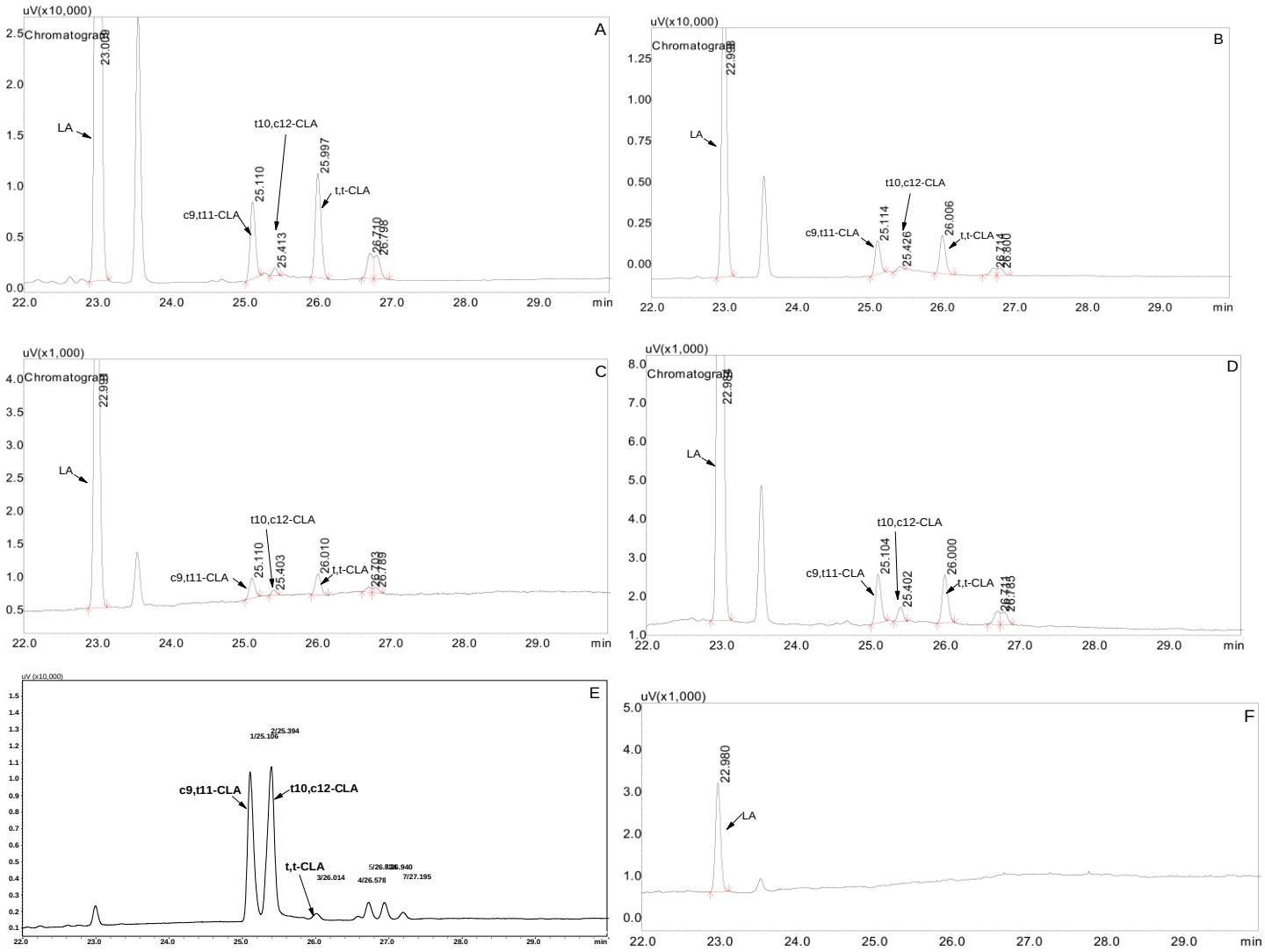

Figure 3. Gas chromatography of assaying isomerase activity after each purification step. (A) Crude enzyme; (B) ammonium sulphate precipitation; (C) DEAE at pH 9.0 (peak I); (D) Sephadex G-100 gel filtration; (E) conjugated linoleic acid, methyl ester Sigma-Aldrich, No. O5632); (F) LA control.

8.0 and 8.5. These results indicate that the proper pH (9.0) could maintain the stability of the isomerase, and combined with a suitable elution buffer (0.12 M NaCl), the active isomerase was obtained. Proteins in the fractions containing the active peak were applied to a Sephadex G-100 gel filtration column after initial desalting and concentrating. Linoleate isomerase activity was retained on the column before elution using PPB. The linoleate isomerase activity of the crude extract and the protein obtained during ammonium sulphate, DEAE and gel filtration was confirmed by GC analysis (Figure 3A to D). And all these fractions was qualified and confirmed by SDS-PAGE.

Efforts to solubilize this enzyme with salt, solvents, detergents and EDTA were unsuccessful due to marked losses in activity or unable to solubilize (Kepler and Tove, 1967). Park et al. (1996) reported the isolation of the *B. fibrisolvens* linoleate isomerase by differential centrifugation and two chromatographic steps. The final preparation appeared to contain a single polypeptide of 19 kDa. The linoleate isomerase from *C. sporogenes* was also purified and characterized, which converted LA to c9, t11 CLA as the major product (Peng et al., 2007). Both the linoleate isomerases from *B. fibrisolvens* and *C. sporogenes* are membrane-bound enzymes. Similar to the linoleate isomerase from *B. fibrisolvens* and *C. sporogenes*, the *L. plantarum* ZS2058 enzyme is membrane-associated. Detergent (Triton X-100) was required for enzyme solubilization, while salt alone was ineffective indicating that the enzyme is likely to be an integral membrane protein. It has long been recognized that *P. acnes* produces t10 and c12 CLA from LA (Verhulst et al., 1987). Purification of the *P. acnes* linoleate isomerase has been achieved (Rosson et al.,

Table 1. Purification of linoleate isomerase from *L. plantarum* ZS2058.

Step	Protein (mg)	Activity[a] (units)	Specific activity (units · mg^{-1})	Yield (%)	Purification[b] (fold)
Crude extract	10.16	201.76	19.86	100	1
Ammonium sulphate precipitation	2.91	149.00	51.13	28.68	2.57
DEAE-Sepharose F.F	1.04	146.43	140.73	10.24	7.09
Gel filtration chromatography	0.83	139.58	168.58	8.15	8.49

[a] "Activity units" are expressed in 1 µg CLA in an hour. [b] Purification fold is the ratio of specific activity measured at each successive step divided by specific activity of the crude extract.

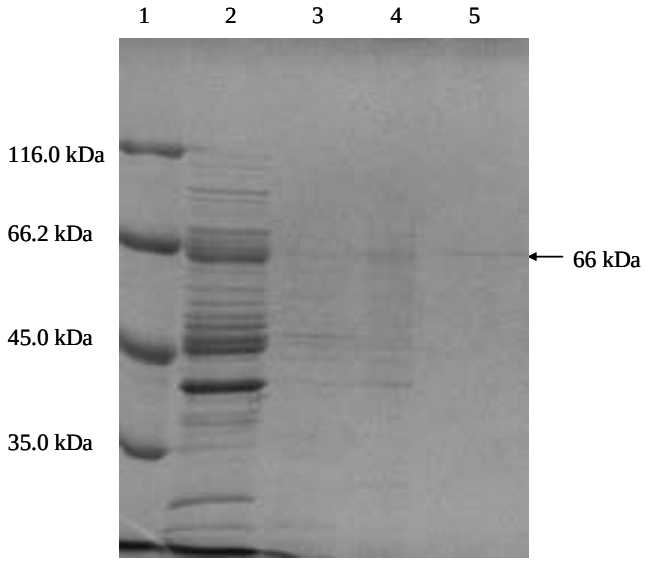

Figure 4. SDS-PAGE pattern of samples from different steps of purification. Lane 1, Protein marker; lane 2, crude extract; lane 3, ammonium sulphate precipitation; lane 4, DEAE fraction; lane 5, gel filtration chromatography.

2001). However, unlike the *B. fibrisolvens* membrane-bound enzyme, the *P. acnes* linoleate isomerase is a soluble protein.

In the gas chromatogram, the peak at 23.000 min was LA, while the peaks at 25.100, 25.400 and 26.000 min were c9, t11 CLA, t10, c12 CLA and t, t CLA, respectively. The fractional conversions of LA for each step were 12.11, 19.43, 8.79 and 9.31% (accounting for area normalization). The results from the different stages of purification are shown in Table 1. SDS-PAGE of the purified isomerase showed a single protein band at 66 kDa (Figure 4), which is equivalent to the molecular weight of this isomerase reported previously. Our results show that *L. plantarum* ZS2058 can isomerize LA, with c9, t11 CLA as a major product. This was also observed in biotransformation experiments using *B. fibrisolvens* cells (Kim et al., 2000). In our previous study, interestingly, significant amounts of t, t CLA were produced by *L. plantarum* ZS2058 cells under anaerobic conditions, while in micro-oxygen conditions these cells produced c9, t11 CLA as the major product. Under aerobic conditions, *L. plantarum* ZS2058 incubated with LA produced c9, t11 CLA and t, t CLA equally. All these results were different from those obtained with *B. fibrisolvens*, *P. acnes* and *C. sporogenes*.

Temperature dependence

The effect of temperature on linoleate isomerase activity was determined at different temperatures. The optimum temperature for enzyme activity was 35°C (Figure 5). Lower or higher temperatures resulted in distinct losses of activity, especially above 45°C. Thus, linoleate isomerase is not resistant to high temperatures.

pH dependence

The effect of pH on enzyme activity was determined using different pH. The optimum pH for linoleate isomerase

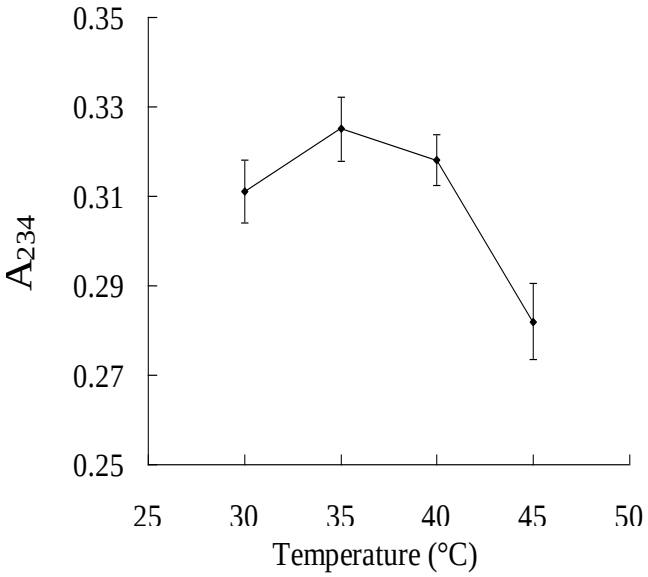

Figure 5. Effect of temperature on activity of linoleate isomerase.

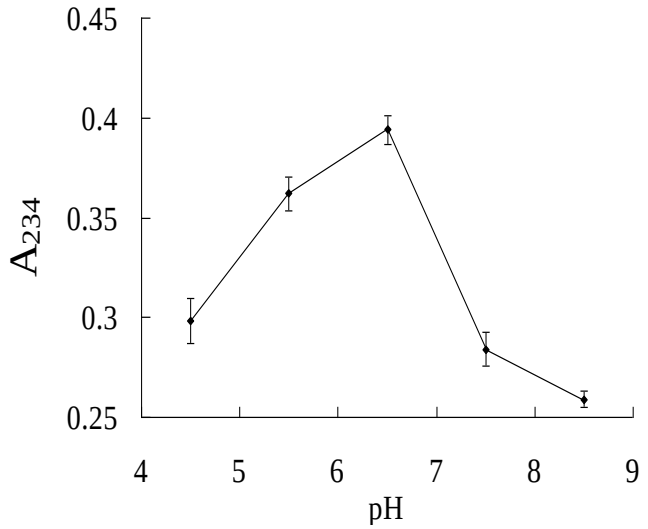

Figure 6. Effect of pH on activity of linoleate isomerase.

activity was determined to be pH 6.5 (Figure 6). Lower or higher pH values resulted in significant losses of activity and so PPB at pH 6.5 was used for all subsequent work. The pH had a strong impact on the linoleate isomerase extraction, but it also affected the stability of the enzyme in crude extracts and in detergent-solubilized form. Best results were obtained using PPB at pH 6.5. *L. plantarum* ZS2058 was highly active at transforming CLAs at pH 6.5 or lower, while CLAs yield decreased dramatically at pH value greater than 7.0. Compared to the linoleate isomerases from *B. fibrisolvens* (Kepler and Tove, 1967), *P. acnes* (Deng et al., 2007), *C. sporogenes* (Peng et al., 2007), and even *L. reuteri* (Rosson et al., 2004), the

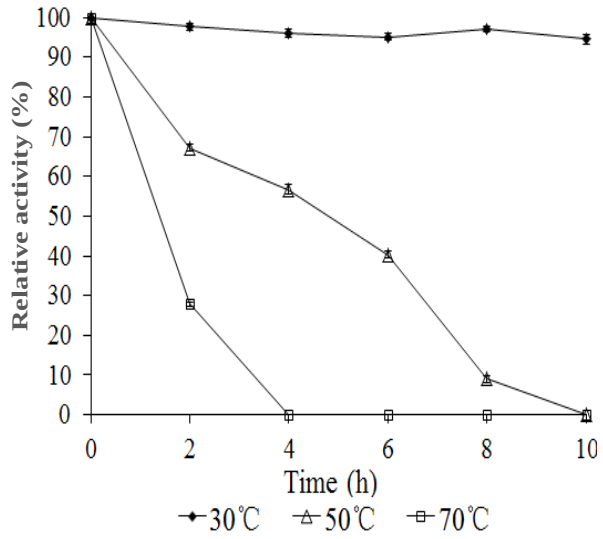

Figure 7. Effect of temperature on stability of linoleate isomerase.

optimal pH reported here for *L. plantarum* is the lowest. The enzyme's pH optimum is close to the optimum pH for the growth of *L. plantarum* (pH 6.0) (Giraud et al., 1991).

Thermostability of enzyme

Since the linoleate isomerase was not resistant to high temperatures, its thermostability was examined. Initial activity was set as 100% at 30°C without incubation. The residue activity was determined after incubating at different temperatures for various times. The results are shown in Figure 7 and these depict that high temperatures resulted in significant losses of activity, especially at 70°C for 4 h when activity was lost completely.

Therefore, the linoleate isomerase was likely denatured at these high temperatures.

Effect of metal ions, metal chelators and cofactors

The various metal ions and metal chelators had no significant influence on linoleate isomerase activity (Table2; Figure 8). In addition, varieties of cofactors at 50 μM (ATP, ADP, NAD, NADH, and NADPH) were also tested for their effects on linoleate isomerase activity. Results indicated that these cofactors did not interfere with the detection of CLA by measurement of A234 and none of these showed any significant effects on enzyme activity (Table 2). The linoleate isomerase was more stable in buffers containing glycerol. It has no additional requirements for reducing agents (NADH, NADPH) or energy compounds (ATP) for its catalytic activity. In our previous study, the linoleate isomerase was also shown to be inhibited at high substrate (LA) concentrations. This result is similar to the membrane-bound linoleate

Table 2. Effect of different metal ions and cofactors on activity of linoleate isomerase.

Metal ion (1 mM)	Enzyme activity (U)	Relative activity (%)
control	66.3 ± 0.75	100.00
Mn^{2+}	71.2 ± 0.15	107.45
Al^{3+}	68.2 ± 1.19	102.94
Pb^{2+}	68.7 ± 3.60	103.60
Ca^{2+}	63.9 ± 1.20	96.38
Mg^{2+}	63.6 ± 0.60	95.93
Cu^{2+}	65.5 ± 1.80	98.87
Fe^{3+}	68.7 ± 0.44	103.60
Fe^{2+}	58.9 ± 5.99	88.93
Ba^{2+}	62.2 ± 0.15	93.91
Zn^{2+}	61.6 ± 5.39	93.00

Cofactors(50 μM)	Enzyme activity (U)	Relative activity (%)
control	65.2 ± 0.15	100.00
ATP	73.5 ± 1.94	112.62
ADP	71.1 ± 0.60	108.95
NAD	64.9 ± 2.24	99.54
NADH	65.2 ± 2.69	100.00
NADPH	66.6 ± 1.94	102.07

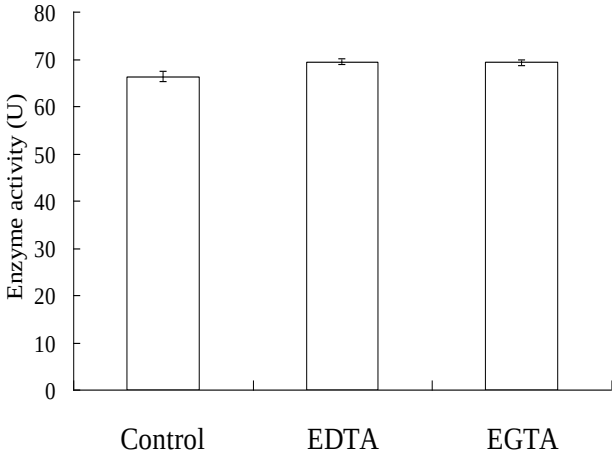

Figure 8. Effect of metal-chelators on activity of linoleate isomerase.

isomerase *from* C. sporogenes *(Peng et al., 2007), but the* mechanism of such inhibition is unclear.

Kinetics parameters

Linoleate isomerase activity was measured at various LA concentrations (0.01 to 0.04 mg/ml) and the production of CLAs was measured using A_{234}. A reciprocal plot of 1/V versus 1/[S] (LA) is presented in Figure 9. Linear regression of enzyme activity at low concentrations confirmed typical Michaelis-Menten behavior. K_m and V_{max} of the enzyme were estimated by fitting the V versus

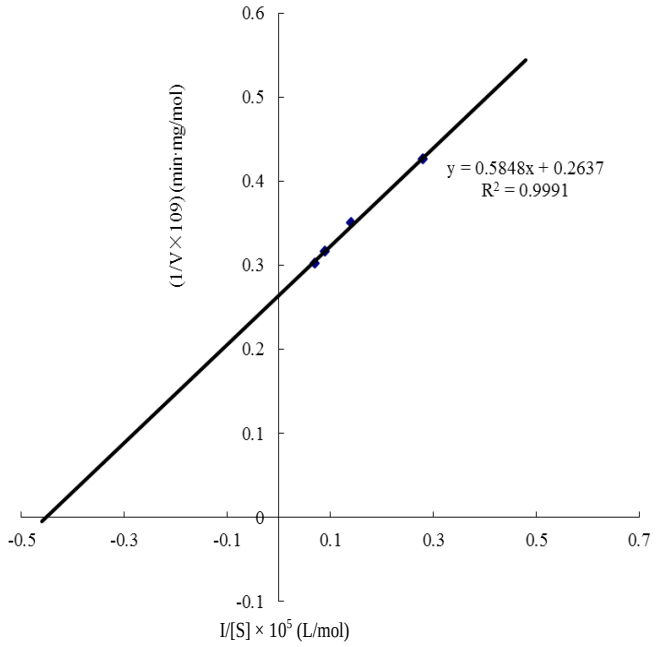

Figure 9. The Lineweaver-Burk pattern of linoleate isomerase.

S data range to the Michaelis-Menten equation. The K_m *of the L. plantarum* ZS2058 linoleate isomerase with LA was 21.5 μM, while the V_{max} was 3.71 nmol/min [(mg· protein)].

Conclusion

A 66-kDa linoleate isomerase of *L. plantarum* ZS2058

was successfully purified and characterized. The optimal pH and temperature were pH 6.5 and 35°C, respectively. For enzyme activity, no external cofactors or energy sources were required, and metal chelators and metal ions had no effect on the activity. This work supports the premise of prevalent occurrence of linoleate isomerase activities in *L. plantarum* and closely related species, and provides useful new data for studying linoleate isomerases from lactic acid bacteria.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (No.20836003), the National High Technology Research and Development Program of China (No.2011AA100901, 2011AA100905), the National Science Fund for Distinguished Young Scholars (31125021) and Fundamental Research Funds for the Central Universities (JUSRP 11017, JUSRP31103).

REFERENCES

Alonso L, Cuesta EP, Gilliland SE (2003). Production of free conjugated linoleic acid by *Lactobacillus acidophilus* and *Lactobacillus casei* of human intestinal origin. J. Dairy Sci. 86(6): 1941-1946.

Ando A, Ogawa J, Kishino S, Shimizu S (2004). Conjugated linoleic acid production from castor oil by *Lactobacillus plantarum* JCM 1551. Enzyme Microb. Technol. 35(1): 40-45.

Banni S (2002). Conjugated linoleic acid metabolism. Curr. Opin. Lipidol. 13: 261-266

Banni S, Heys CSD, Wahle KWJ (2003). Conjugated linoleic acid as anticancer nutrients: Studies *in vivo* and cellular mechanisms. In: Sebedio J, Christie WW and Adolf R (Eds). Advances in conjugated linoleic acid research. 2: 267-282. AOCS Press, Champaign, Il.

Belury MA (2002). Dietary conjugated linoleic acid in health: Physiological effects and mechanisms of action. Annu. Rev. Nutr. 22: 505-531.

Belury MA (2003). Conjugated linoleic acids in type 2 diabetes mellitus: Implications and potential mechanisms. In: Sebedio J, Christie WW and Adolf R (Eds). Advances in conjugated linoleic acid research. 2: 302-315. AOCS Press, Champaign, Il.

Bligh EG, Dyer WJ (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37(8): 911-917.

Chin SF, Liu W, Storkson JM, Ha YL, Pariza MW (1992). Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinagens. J. Food Comp. Anal. 5:185-197.

Deng MD, Grund AD, Schneider KJ, Langley KM, Wassink SL, Peng SS, Rosson RA (2007). Linoleic acid isomerase from *Propionibacterium acnes*: Purification, characterization, molecular cloning, and heterologous expression. Appl. Biochem. Biotechnol. 143(3): 199-211.

Giraud E, Lelong B, Raimbault M (1991). Influence of pH and initial lactate concentration on the growth of *Lactobacillus plantarum*. Appl. Microbiol. Biotechnol. 36: 96-99.

Griinari JM, Bauman DE (1999). Biosynthesis of conjugated linoleic acid and its incorporation into meat and milk in ruminants. In: Yurawecz MP, Mossoba MM, Kramer JKG, Pariza MW and Nelson GJ (Eds). Advances in conjugated linoleic acid research. 1: 180-200. AOCS Press, Champaign, Il.

Haas MH, Kramer JKG, McNeill G, Scott K, Foglia TA, Sehat N, Fritsche J, Mossoba MM, Yurawecz MP (1999). Lipase-catalyzed fractionation of conjugated linoleic acid. Lipids 34(9): 979-987.

Kepler CR, Hirons KP, Mcneill JJ, Tove SB (1966). Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. J. Biol. Chem. 241(6): 1350-1354.

Kepler CR, Tove SB (1967). Biohydrogenation of unsaturated fatty acids. III. Purification and properties of a linoleate 12-cis, 11-trans-isomerase from *Butyrivibrio fibrisolvens*. J. Biol. Chem. 242(24): 5686-5692.

Kim YJ, Liu R, Hai, Bond DR, Russell JB (2000). Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrosolvens* A38. Appl. Environ. Microbiol. 66(12): 5226-5230.

Kritchevsky D, Tepper SA, Wright S, Czarnecki SK, Wilson TA, Nicolosi RJ (2004). Conjugated linoleic acid isomer effects in atherosclerosis: Growth and regression of lesions. Lipids, 39(7): 611-616.

Lee SO, Kim CS, Cho SK, Choi HJ, Ji GE, Oh DK (2003). Bioconversion of linoleic acid into conjugated linoleic acid during fermentation and by washed cells of *Lactobacillus reuteri*. Biotechnol. Lett. 25(12): 935-938.

Lin TY, Lin CW, Lee CH (1999). Conjugated linoleic acid concentration as affected by lactic cultures and added linoleic acid. Food Chem. 67(1): 1-5.

Lin TY, Lin CW, Wang YJ (2002). Linoleic acid isomerase activity in enzyme extracts from *Lactobacillus acidophilus* and *Propionibacterium freudenreichii*. J. Food. Sci. 67(4): 1502-1505.

Mounts TL, Dutton HJ, Glover D (1970). Conjugation of polyunsaturated acids. Lipids 5(12): 997-1005.

Ogawa J, Kishino S, Ando A, Sugimoto S, Mihara K, Shimizu S (2005). Production of conjugated fatty acids by lactic acid bacteria. J. Biosci. Bioeng. 100(4): 355-364.

Ogawa J, Matsumura K, Kishino S, Omura Y, Shimizu S (2001). Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. Appl. Environ. Micobiol. 67(3): 1246-1252.

Pariza MW, Park Y, Cook ME (1999). Conjugated linoleic acid and the control of obesity and cancer. Toxicol. Sci. 52 (2 Suppl): 107-110.

Pariza MW, Park Y, Cook ME (2000). Mechanism of action of conjugated linoleic acid: Evidence and speculation. Proc. Soc. Exp. Biol. Med. 223(1): 8-13.

Park SJ, Park KA, Park CW, Park WS, Kim JO, Ha YL (1996). Purification and amino acid sequence of the linoleate isomerase produced from *Butyrivibrio fibrisolvens* A-38. J. Food Sci. Nutr. 1: 244-251.

Peng SS, Deng MD, Grund AD, Rosson RA (2007). Purification and characterization of a membrane-bound linoleic acid isomerase from *Clostridium sporogenes*. Enzyme. Microb. Tech. 40(4): 831-839.

Peterson GL (1983). Determination of total protein. Methods Enzymol. 91: 95-121.

Rainio A, Vahvaselka M, Suomalainen T, Laakso S (2001). Reduction of linoleic acid inhibition in production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. Can. J. Microbiol. 47(8): 735-740.

Rainio A, Vahvaselka M, Suomalainen T, Laakso S (2002). Production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. Lait, 82: 91-101.

Rosson RA, Deng MD, Grund AD, Peng SS (2001). PCT WO0100846-A.

Rosson RA, Ground AD, Deng MD, Sanchez-Riera F (2004). United States Patent US 6,743,609 B1

Verhulst A, Janssen G, Parmentier G, Eyssen H (1987). Isomerization of polyunsaturated long chain fatty acids by *Propionibacteria*. Syst. Appl. Microbiol. 9: 12-15.

Verhulst A, Semjen G, Meerts U, Janssen G, Parmentier G, Asselberghs S, Van Hespen H, Eyssen H (1985). Biohydrogenation of linoleic acid by *Clostridium sporogenes*, *Clostridium bifermentans*, *Clostridium sordellii* and *Bacteroides* sp. FEMS Microbiol. Lett. 31(4): 255-259.