Appl Biochem Biotechnol (2007) 143:199–211
DOI 10.1007/s12010-007-8075-1

Linoleic Acid Isomerase from *Propionibacterium acnes*: Purification, Characterization, Molecular Cloning, and Heterologous Expression

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Accepted: 28 September 2007 / Published online: 27 October 2007
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Abstract *Propionibacterium acnes* strain ATCC 6919 catalyzes the isomerization of the double bond at the C9 position in linoleic acid (*c*9*,c*12, 18:2) to form *t*10,*c*12 conjugated linoleic acid (CLA, 18:2). CLA has significant health benefits in animal and human. The linoleic acid C9 isomerase was purified to an apparent homogeneity by successive chromatography on diethylaminoethyl (DEAE) anion exchange, hydrophobic interaction, and chromatofocusing columns. Two degenerated oligonucleotide primers were synthesized according to the N-terminal peptide sequence to clone, by polymerase chain reaction (PCR), a short nucleotide sequence (62 bp) of the isomerase gene. The linoleic acid isomerase gene (*lai*) was subsequently cloned by inverse PCR. The amino acid sequence deduced from the *lai* coding sequence predicts a protein of 424 amino acid residues (48 kDa), excluding the N-terminal methionine, which was absent in the polypeptide purified from the native host. The isomerase shares no significant sequence homology to other enzymes except a flavin-binding domain in the N-terminal region. The recombinant isomerase purified from *Escherichia coli* showed a typical ultraviolet spectrum for FAD-bound proteins. The recombinant enzyme produced a single isomer of *t*10,*c*12-CLA from linoleic acid, as demonstrated by gas chromatography and gas chromatography-mass spectrum analysis. The recombinant isomerase protein was expressed at high levels in *E. coli*, but it was almost totally sequestered in inclusion bodies. The level of active isomerase was increased 376-fold by medium and process optimization in bench-scale fermentors.

Keywords Biotransformation · Chromatography · Cloning · Conjugated linoleic acid · Expression · Fermentation · Linoleic acid isomerase

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Introduction

Conjugated linoleic acid (CLA, 18:2), an octadecadienoic acid with two conjugated double bonds, has a variety of positional and geometric isomers. Ha et al. [1] discovered anticarcinogenic effects of CLA in mouse epidermal neoplasia. Since then, numerous publications have demonstrated various biological and physiological benefits of CLA in animal and human health, such as inhibiting the initiation of carcinogenesis and tumorigenesis, reducing atherosclerosis, improving hyperinsulinemia, altering the low density lipoprotein/high density lipoprotein cholesterol ratio, and reducing body fat, while increasing muscle mass [2, 3]. Although CLA is present in virtually all food, especially diary products and other food derived from ruminant animals, this important nutrient may not be provided at adequate quantity in human diet [4].

CLA is currently available as a dietary supplement, and it is produced by alkaline isomerization of linoleic acid (LA, *c*9,*c*12 octadecadienoic acid, 18:2) or vegetable oils rich in LA esters. Chemical isomerization results in predominantly *c*9,*t*11 and *t*10,*c*12 isomers (40–45% each) with a variety of other isomers [5, 6]. Recent studies have demonstrated separate and/or synergistic actions of the two biologically active isomers, *c*9,*t*11 and *t*10, *c*12 CLA [3]. Therefore, single CLA isomers or isomers formulated at specific ratios are possibly the preferred compositions. Processes to prepare single isomers from chemically synthesized mixtures are not practical at large scale because of high cost of purification and difficulties in achieving high purity [7–9].

LA isomerases catalyze the conversion of LA into single CLA isomers, *c*9,*t*11 or *t*10,*c*12 CLA, depending on the specific enzyme (Fig. 1). Tove's group described a LA C12 isomerase that catalyzed the conversion of LA into *c*9,*t*11 CLA, an intermediate step in the biohydrogenation of LA to stearic acid in anaerobic rumen bacterium *Butyrivibrio fibrisolvens* [10–12]. The LA C12 isomerase has since been detected in a variety of bacteria [13–16]. Biotransformation of LA using microbial cells and enzyme extracts has been explored for the production of *c*9,*t*11 CLA [17, 18]. *Propionibacterium acnes* and *Megasphaera elsdenii* were reported to contain a LA C9 isomerase converting LA to *t*10, *c*12 CLA [19, 20]. The present paper describes purification and cloning of the *P. acnes* LA C9 isomerase. To the best of our knowledge, this is the first LA isomerase gene that has

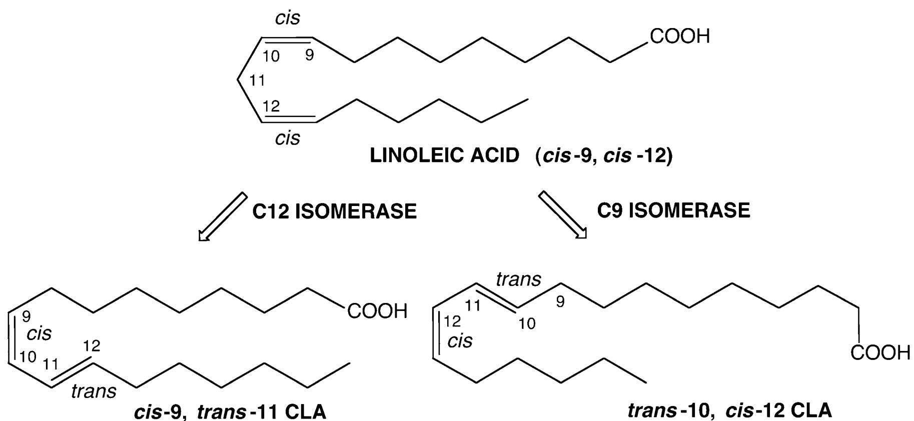

Fig. 1 Enzymatic conversion of LA to *c*9,*t*11 CLA by LA C12 isomerase and to *t*10,*c*12 CLA by LA C9 isomerase

been cloned and functionally expressed. Efficient enzymatic processes can now be developed for CLA production using the recombinant technology.

Material and Method

Bacterial Strains and Growth Media

P. acnes ATCC 6919, a strict anaerobic bacterium, was purchased from American Type Culture Collection (ATCC). It was grown anaerobically without agitation at 37 °C in Difco Brain–Heart-Infusion Broth (BHI) (Becton, Dickinson and Company, Sparks, MD) in closed containers with limited head space. *Escherichia coli* strains were grown aerobically in Luria–Bertani (LB) broth with appropriate antibiotic selection.

LA Isomerase Assay

LA isomerase activity was assayed by biotransformation of LA using whole cell suspension or enzyme extracts as described previously [16]. A unit of isomerase activity is defined as the amount of enzyme forming 1 μmol CLA per minute at room temperature.

GC and GC-MS analysis

Fatty acids were converted to fatty acid methyl esters (FAMES) as described previously [16]. To identify the products of isomerase reactions, FAMES were analyzed on a HP 6890 model gas chromatograph (GC) fitted with a flame ionization detector. The detector and injector were held at 250 °C. After splitless injection, the column (Supelco SP-2380, 100 m, 0.25 mm ID) was held at 155 °C for 15 min, followed by an increase to 180 °C at a rate of 1 °C/min. After a 30-min hold at 180 °C, the temperature was increased to 220 °C at 10 °C/min, and held at 220 °C for 5 min. CLA standards were kindly provided by Dr. Michael W. Pariza's laboratory (University of Wisconsin, Madison), and also obtained from Sigma (St. Louis, MO). CLA peak identification was based on chromatograms provided by Dr. Pariza. In routine GC analysis to determine isomerase activity, FAMES were analyzed on a 30-m Supelco WAX-10 column (0.53 mm I.D., 1 μm thickness) using an isothermal program at 215 °C [16].

2-Alkenyl-4,4-dimethyloxazoline (DMOX) derivatives were formed by refluxing fatty acids with 500 μl of 2-amino-2-methyl-1-propanol under nitrogen for 18 h at 160 °C. After cooling, 5 ml water and 1 ml hexane were added and mixed by shaking. The hexane layer was analyzed using a HP 5890A gas chromatograph fitted with a HP 5970 MS quadropole mass spectrometer (GC-MS). The injector and detector were held at 300 °C. Splitless injection was made onto a Restec Rtx-5MS column (15 m, 0.25 mm ID). The oven temperature was initially set at 150 °C, increased to 250 °C at 10 °C /min, and held at 250 °C for 10 min.

Enzyme Kinetics Studies

LA isomerase activity was assayed in a quartz cuvette (1-cm light-path with a magnetic stirrer) at room temperature using a HP 8452A diode array spectrophotometer, essentially as described previously [11]. The cuvette was filled with 1 ml of reaction mixture containing 0.1 M potassium phosphate, pH 7.5, 10 mM NaCl, 10% 1,2-propanediol, varying concentrations of LA, and other additions as indicated. The isomerization reaction was

initiated by adding l0 μl of purified isomerase (about 0.1 μg). Changes in absorbance at 234 nm (characteristic of conjugated double bonds in fatty acids) during the first minute were used to calculate reaction rates using an extinction coefficient of 2.4×10^4 M^{-1} cm^{-1} [11].

LA Isomerase Extraction

To monitor isomerase activity during extraction and purification, CLA formed in biotransformation reactions was extracted with 1 ml of hexane and quantified by measuring absorbance at 234 nm on a HP 8452A diode array spectrophotometer. Cell cultures from 2-l bottles were harvested by centrifugation at 10,000×*g* for 30 min at room temperature. Cells were washed once with a bis–Tris buffer (0.1 M, pH 6.0) containing 0.9% NaCl. Cells were used immediately or stored at −80 °C. Cell paste was suspended in the same buffer and passed twice through a 40-k French pressure cell (SLM Instruments, Inc., Urbana, IL) at the maximal working pressure of 40,000 psi. After removing cell debris and unbroken cells by centrifugation at 15,000×*g* for 30 min, the crude extract was cleared by ultracentrifugation at 45,000×*g* for 90 min. The supernatant retaining over 93% of the activity detected in the crude extract.

LA Isomerase Purification

P. acnes LA isomerase was purified by successive chromatography on diethylaminoethyl (DEAE) column, hydrophobic interaction column (HIC), and chromatofocusing column. The supernatant from the ultracentrifugation step of the isomerase extraction was dialyzed against a buffer composed of 0.1 mM Tris pH 6.0 and 1 mM dithiothreitol, and applied onto a DEAE-5PW column (2.1×15 cm, Tosohaas, Gmbh, Stuttgart, Germany). Elution was started with a linear gradient from 0 to 0.175 M NaCl and continued with 70 ml of 0.175 M NaCl. After the elution of the LA isomerase at this salt concentration, the salt gradient continued to increase to 1 M to elute other proteins. Fractions from several runs containing significant LA isomerase activity were pooled, dialyzed, applied onto one column, and eluted as described above. The active fractions were pooled, and made 1 M in $(NH_4)_2SO_4$. The sample was applied to a phenyl HIC (Tosohaas, Phenyl-5PW, 21.5 mm×15 cm) in several runs. The isomerase bound very tightly to the column and was eluted using an ethylene glycol gradient from 5 to 30%. Selected fractions from HIC were pooled, concentrated, dialyzed against the chromatofocusing column loading buffer (20 mM bis–Tris, pH 6.5), and applied to a Pharmacia monoP chromatofocusing column PBE94 pre-equilibrated with the loading buffer. A pH gradient of 5.5 to 4.0 was formed using 10% Polybuffer 74 (pH 4.0). The isomerase activity was eluted in a sharp peak around pH 4.2. Active fractions were examined for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining. Several fractions appeared to contain a single protein band of approximately 50 kDa in size. The N-terminal peptide sequence was determined using the purified LA isomerase protein.

PCR Reaction with Degenerated Oligonucleotide Primers

Degenerated oligonucleotide primers (Table 1) were designed according to the N-terminal peptide sequence of the purified LA isomerase. Forward primer PAo5 was based on residues 8 through 14. Reverse primer PAo11 corresponded to residues 22 through 28. PCR reactions (100 μl) contained 4 dNTP (0.1 mM each), primer PAo5 (5 μM), primer PAo11

Table 1 Oligonucleotide primers used in PCR reactions.

Primer name	Sequence	Application
PAo5	5'-ATC GCS ATN ATN GGN GCN GG-3'	Degenerated forward primer for cloning the N-terminal peptide coding sequence
PAo11	5'-CC NGC YTG CCA NAR RTA CAT-3'	Degenerated reverse primer for cloning the N-terminal peptide coding sequence
PAo16	5'-GGG CCA GCC CCY ATN AT-3'	Forward primer for the first round inverse PCR
PAo17	5'-GCT GGC TGC CGG AAT GTA-3'	Reverse primer for the first round inverse PCR
PAo21	5'-CGA TGT CGG CGT GGT AC-3'	Forward primer for the second round inverse PCR
PAo22	5'-TCA CGT ATC GCC ATC ATC-3'	Reverse primer for the second round inverse PCR
PAo23	5'-AAT CCG GCC TGT TCG AG-3'	Forward primer for the second round inverse PCR
PAo24	5'-AGG ACG GCG AGA TCT AC-3'	Reverse primer for the second round inverse PCR
PAo41	5'-CAGA *CAT ATG* TCC ATC TCG AAG GAT TC-3'	Forward primer for cloning the LA isomerase CDS, containing an *Nde*I site
PAo42	5'-CTAT *CTC GAG* TCA CAC GAA GAA CCG CGT C-3'	Reverse primer for cloning the LA isomerase CDS, containing an *Xho*I site
PAo44	5'-CTAT *CTC GAG* CAC GAA GAA CCG CGT C-3'	Reverse primer for cloning the isomerase CDS to express the LA isomerase with a C-terminal His tag, containing an *Xho*I site

N=A, C, G or T; S=G or C; Y=C or T; R=A or G. Restriction sites added to the primer sequences are italicized.

(5 μM), *P. acnes* genomic DNA (1.4 μg), and Taq polymerase (0.5 μl) in the buffer provided by the polymerase vendor (Qiagen, Madison, WI). Thermal cycling conditions were: 7 min at 95 °C, 5 min at 54–65 °C, 1 min at 72 °C; 40 cycles of 1 min at 95 °C, 1 min at 54–65 °C, and 1 min at 72 °C;10 min at 72 °C. A PCR product of the expected size (62 bp) was produced using annealing temperature of 54 °C. The PCR product was isolated from an agarose gel and cloned into pCR-Script SK (+) Amp (Stratagene Cloning System, La Jolla, CA).

Inverse PCR

Primers PAo16 and PAo17 were synthesized according to the 62-bp PCR product for cloning the isomerase gene by inverse PCR. *P. acnes* genomic DNA were digested with different restriction enzymes or pairs of enzymes that have compatible ends. DNA digests were circularized with T4 DNA ligase and used as DNA templates in PCR reactions. A PCR product of about 570 bp was generated with a *Bam*HI digest. The PCR product was cloned and sequenced. The fragment contained 5' upstream sequence and a portion of the coding sequence (311 bp), with the C-terminus coding sequence missing.

Southern Blot Analysis of *P. acnes* Genomic DNA

P. acnes genomic DNA digested with different restriction enzymes was analyzed by Southern Blot using as probe the 570-bp sequence labeled with biotinylated nucleotides (NEBlot Phototope kit, New England Biolabs). For enzymes that were absent in the 570-bp sequence (*Pvu*II and *Xho*I), only one hybridization band was detected on Southern Blot. The DNA band in the *Xho*I digest was about 2.8 kb. The 570-bp sequence contained unique sites for *Bgl*I, *Fok*I, and *Sal*I. Two hybridization bands were detected in these DNA digests. The hybridization bands in the *Bgl*I digest were about 2.5 and 3.5 kb.

Second Round Inverse PCR

P. acnes genomic DNA was digested with either *Bgl*I or *Xho*I, circularized and used as the template DNA in inverse PCR reactions. Primers PAo21and PAo22 were designed to clone the 5' *Bgl*I fragment; and primers PAo23 and PAo24 to clone the 3' *Bgl*I fragment. Primer PAo21 and PAo24 were also used in PCR reactions to amplify the *Xho*I fragment. The PCR amplification with the *Bgl*I digest generated a PCR product of about 2.5 kb using the primers PAo21 and PAo22, but no PCR product of the expected size was produced using primers PAo23 and PAo24. A PCR product of about 2.5 kb was amplified from the *Xho*I digest using the primer pair PAo21/PAo24. Both PCR products were cloned and sequenced. As a result, a total of 5.5 kb was cloned from the locus of the LA isomerase gene.

LA Isomerase Expression in *E. coli*

The LA isomerase coding sequence was amplified by PCR from *P. acnes* genomic DNA using forward primer PAo41 (containing an *Nde*I site) and reverse primers PAo42 or PAo44 (both containing an *Xho*I site). Primer PAo42 contained nucleotides reverse complementary to C-terminal coding sequence and the stop codon, while primer PAo44 was designed for expression of a LA isomerase with a C-terminal His tag. PCR products of the expected size (1.3 kb) were digested with *Nde*I and *Xho*I and ligated to vector pET24a(+) (Novagen) that was predigested with the same enzymes. The ligated DNA was transformed into *E. coli* One Shot TOP 10 cells (Invitrogen, Madison, WI). Recombinant plasmids were transformed into *E. coli* strain BL21 (DE3) (Novagen).

Fifty milliliters of LB broth containing kanamycin (50 μg/ml) was inoculated (1:100, *v*/*v*) with a freshly grown overnight culture of *E. coli* BL21 (DE3) hosting an isomerase expression plasmid. After growing at 37 °C for 3 h, cultures were induced with 50 to 1,000 μM isopropylthio-β-D-galactoside (IPTG) for 2 h at 26 °C.

Cultures were harvested by centrifugation at 10,000×*g* for 10 min at 4 °C. Cells were suspended into 5 ml of a breakage buffer (100 mM Tris–HCl, pH 7.5, 10 mM NaCl, 10% glycerol). Cells were broken by passing through a 10-k French pressure cell (SLM Instruments, Inc., Urbana, IL) at the maximal working pressure of 10,000 psi. The cell lysate was assayed for isomerase activity by biotransformation of LA.

Analysis of the Recombinant LA Isomerase

E. coli cell lysate was centrifuged 10,000×*g* at 4 °C for 30 min to separate soluble protein (supernatant) from inclusion bodies (pellet). The total lysate and soluble protein were analyzed under denaturing conditions on NuPAGE 4–12% Bis–Tris–gels (Invitrogen). The LA isomerase with a C-terminal His tag was isolated from inclusion bodies and purified by using a His.Bind resin column (Novagen, Madison, WI) following the vendor's instructions. The purified fusion protein was used to immunize rabbits. LA isomerase in soluble protein was detected by Western Blot using the rabbit antiserum raised against the isomerase. Blots were scanned and analyzed to determine the relative density of the isomerase protein band using BioImage System (UVP Inc, Upland, CA).

LA Isomerase Production in 14-l Fermentors

Fed-batch fermentation was run in 14-l fermentors. The optimized medium contained (per liter): 2 g $(NH_4)_2SO_4$, 1.6 g KH_2PO_4, 9.9 g $Na_2HPO_4 \cdot 7H_2O$, 0.65 g Na_3 citrate$\cdot 2H_2O$,

0.49 g $MgSO_4 \cdot 7H_2O$, 20 g N-Z Amine, 20 g glucose, 5 ml antifoam agent DOW 1520, and 50 mg kanamycin. The pH of the medium was about 7.0. All components except glucose and kanamycin were batch sterilized in fermentors. A glucose solution of 52% (*w*/*v*) was sterilized separately and added with kanamycin into the medium before inoculation.

A seed culture was started by inoculating 0.9–1.0 ml of a frozen overnight culture into 500 ml LB broth containing 50 mg/l kanamycin in a 2-l flask. The culture was shaken at 180–200 rpm at 37 °C for 17 h. Fermentors containing 10 l of medium were inoculated with 250 to 500 ml of seed culture. Initial aeration rate of 2.0 lpm (liter per min) was provided by agitation at 150 rpm. Agitation rate was increased to maintain dissolved oxygen level above 5–10%. When OD_{600} of 10 was reached, IPTG was added to 1 mM to induce LA isomerase expression. Temperature was maintained at 37 and 26 °C before and after induction, respectively. pH was controlled at 6.8–7.0 with anhydrous ammonia for growth and controlled at 7.0–7.2 with feeding of a 52% (*w*/*v*) glucose solution (total, 50 g glucose/l) after induction. Fermentation was discontinued 46 h after inoculation.

Results

Characterization of the *P. acnes* LA C9 Isomerase

Incubation of LA with whole cells of *P. acnes* ATCC 6919 or crude enzyme extracts generated *t*10,*c*12-CLA as reported previously [19]. No other CLA isomers were detected. When a crude extract was fractionated by ultracentrifugation at 45,000×*g* for 90 min, the supernatant retained over 93% activity. Therefore, the *P. acnes* LA C9 isomerase is clearly a soluble protein, unlike LA C12 isomerases from *B. fibrisolvens* [12], *Lactobacillus reuteri* [14], and *Clostridium sporogenes* [16], which have been shown to be membrane proteins (or enzymes tightly bound to membranes).

P. acnes LA C9 isomerase activity in the crude soluble enzyme preparation was not significantly affected by overnight dialysis. Addition of 50 μM of NAD, NADH, NADP, NADPH, FAD, FMN, ADP, ATP, and glutathione did not affect LA isomerase activity (data not shown). No effects were observed with calcium chloride (5 mM), magnesium sulfate (10 mM), EDTA (5 mM), 1,10-phenanthroline (1 mM), sulfhydryl reagent *p*-chloromercuribenzoate (5 μM) or N-ethylmaleimide (100 μM). LA isomerase activity was about the same at room temperature and 37 °C, but very low at 4 °C. Levels of CLA production was positively correlated to substrate concentrations when the enzyme extract was incubated with 36, 90, 357, 892, and 1,786 μM LA. Moreover, the amount of CLA produced continued to increase over time for at least 1.5 h (data not shown). These data clearly indicate that the *P. acnes* LA isomerase is not subject to product inhibition. Again, this is in a sharp contrast to LA C12 isomerases, which were inhibited by LA above 20 μM [12, 14, 16].

LA Isomerase Purification

P. acnes LA isomerase was purified 147-fold by successive chromatography on DEAE, HIC, and chromatofocusing columns (Table 2). The purified enzyme was a single protein band on SDS-PAGE with an estimated molecular weight of about 50 kDa. The N-terminal sequence of 35 amino acid residues was determined: SISKD SRIKI IGAGP AGLAA GMYLW QAGFX DYTIL

The initiating methionine residue was apparently removed enzymatically, as it was missing at the N-terminus. Kinetic parameters of the *P. acnes* LA isomerase were determined using the

Table 2 Purification of the linoleate C9 isomerase from *P. acnes*.

Step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (fold)
Crude extract	419	1,65	3.26	100	1
DEAE column	34.8	774	22.2	56.7	7
Hydrophobic interaction column	1.83	250	137	18.3	42
Chromatofocusing column	0.107	51	478	3.8	147

purified enzyme and compared in Table 3 to values reported for other LA isomerases [12, 14, 16]. The V_{max} value of the *P. acnes* LA isomerase was lower than the *L. reuteri* enzyme but comparable to the *C. sporogenes* enzyme. *P. acnes* LA isomerase had a higher K_m for LA than other isomerases. Both LA C9 and C12 isomerases have a similar optimal pH of 7.2 to 7.5.

Molecular Cloning and Characterization

Degenerated nucleotide primers were synthesized for PCR according to residues 8 through 14 (forward primer) and 22–28 (reverse primer) of the N-terminal sequence of the purified LA isomerase. The PCR product of the expected size (62 bp) was cloned and sequenced. Based on this sequence, primers were synthesized for inverse PCR, leading to the cloning of a larger portion of the isomerase gene (570 bp). After a second round of inverse PCR, a DNA region of 5.5 kb was cloned. It contains the entire LA isomerase gene (*lai*) and three flanking ORFs, two located upstream (A and B) and one located downstream (C) of *lai*. ORF-B, -C, and the isomerase gene are coded on the same DNA strand, while ORF-A is on the complementary strand. A transcription terminator-like sequence was located at positions 33 to 69 down stream of the stop codon of the isomerase ORF. However, no obvious terminator or promoter sequences were found between the ORF-B and the isomerase gene. It was not determined if the isomerase and this ORF are co-transcribed. Southern Blot analysis using the 570-bp sequence as probe indicates that the *P. acnes* genome contains a single *lai* gene.

The isomerase ORF encodes for a protein of 424 residues, excluding the initiating methionine residue. The amino acid sequence deduced from the nucleotide sequence agreed with the N-terminal peptide sequence of the purified enzyme except at position 25, where it is a glutamate in the former and a tryptophan in the latter. The predicted molecular weight of the LA isomerase is 48 kDa, in reasonably good agreement with the molecular weight of 50 kDa

Table 3 Characteristic of linoleate isomerase from different microorganisms.

Organism	CLA isomer	Optimal pH	K_m (μM)	V_{max} (nmol/min/mg)	Enzyme solubility	Substrate inhibition
P. acnes	t10,c12	7.3	17.2	478	soluble	No
L. reuteri[a]	c9,t11	7.5	8.1	880	Membrane bound	Yes
C. sporogenes[b]	c9,t11	7.5	11.9	548	Membrane bound	Yes
B. fibrisolvens[c]	c9,t11	7.2	9.7	38	Membrane bound	Yes

[a] Purified enzyme [14]

[b] Purified enzyme [16]

[c] A particulate preparation of the isomerase [12]

estimated for the native enzyme. No additional in-frame ATG codon exists upstream of the putative ATG start codon. Moreover, a ribosome binding site-like sequence (AGGAAG) was found four nucleotides upstream of the putative translation initiation codon of the ORF.

The *P. acnes* isomerase shares little homology with other proteins except for a N-terminal domain that contains the consensus sequence of a dinucleotide binding motif shared by a large number of FAD containing proteins such as protoporphyrinogen oxidases, monoamine oxidases, and phytoene desaturases. The consensus sequence for the dinucleotide binding motif is: U_4G(G/A)GUXGL(X_2)(A/S)(X_2)L($X_{6–12}$)UX(L/V)UE(X_4) UGG($X_{9–13}$)(G/V)(X_3)(D/E)XG, where X stands for any residue and U stands for a hydrophobic residue [21]. The *P. acnes* genome has been recently sequenced, and the *lai* ORF was annotated as a putative aminooxidase [22].

Functional Expression of the Cloned LA Isomerase Gene

The *P. acnes* LA isomerase coding sequence was subcloned into a pET vector and the enzyme expressed in *E. coli.* Incubation of LA with the recombinant LA isomerase formed products with the UV absorbance peak characteristic for CLA. The products were further analyzed by GC and GC-MS. The separation of a typical commercial CLA mixture is shown in Fig. 2a. Products generated by the recombinant LA isomerase contained the substrate and a major peak of *t*10,*c*12 (Fig. 2b). To further elucidate the double bond positions in CLA produced by the recombinant enzyme, a DMOX derivative was made and analyzed by GC-MS. The fragmentation pattern clearly demonstrates unsaturations at positions 10 and 12 (Fig. 3).

The LA isomerase was also expressed in *E. coli* with a C-terminal His tag. The tagged LA isomerase purified using a nickel column was yellowish and displayed a typical UV spectrum of FAD-bound proteins (data not shown). The purified recombinant isomerase

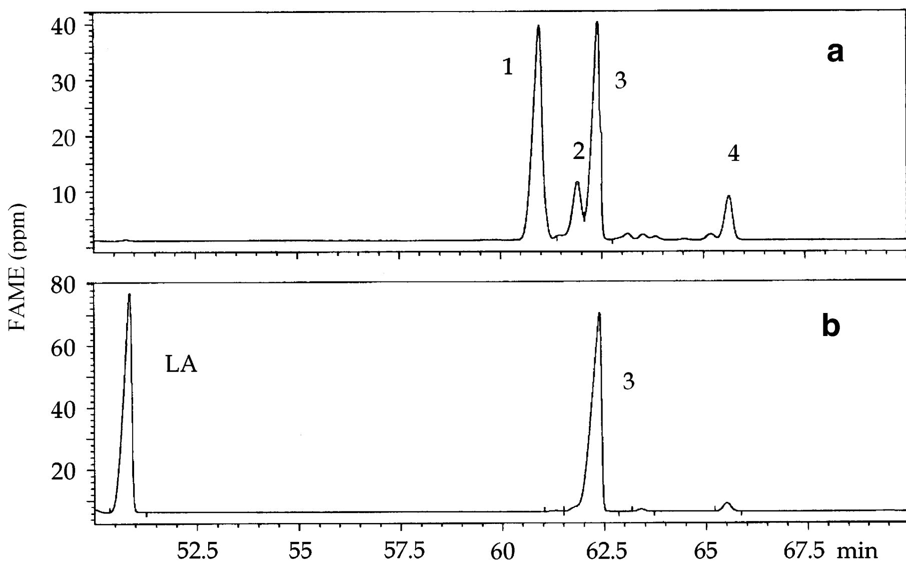

Fig. 2 GC analysis of fatty acid methyl esters (FAMES) of LA and CLA using a 100-m supelco SP-2380 column. **a** A commercial sample of chemically synthesized CLA. Four major peaks are indicated: peak 1: *c*9, *t*11-CLA, peak 2: *c*9,*c*11-CLA plus *c*10,*c*12-CLA, peak 3: *t*10,*c*12-CLA, and peak 4: *t*9,*t*11-CLA plus *t*10, *t*12-CLA. **b** CLA formed by the recombinant LA C9 isomerase

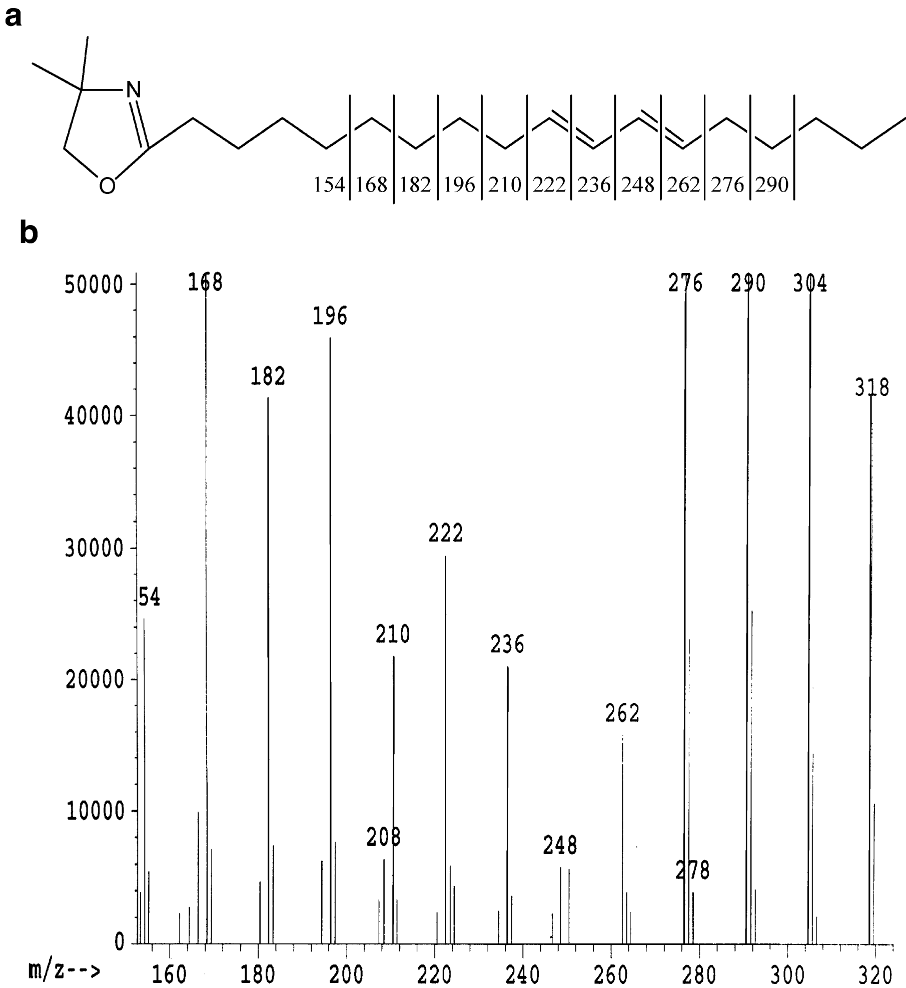

Fig. 3 GC-MS spectrum of the DMOX (2-alkenyl-4, 4-dimethyloxazoline) derivative of CLA produced by using *P. acnes* LA C9 isomerase expressed in *E. coli*. **a** fragmentation patterns expected for *t*10,*c*12 CLA; **b** Mass spectrum observed with the CLA product

was injected into rabbits to raise antibodies. The recombinant LA isomerase protein with or without the His tag was expressed as a predominant protein band as detected by SDS-PAGE after induction with 1 mM IPTG at 37 °C (data not shown). However, the recombinant protein was mostly sequestered in inclusion bodies and LA isomerase activity in enzyme extracts was very low, typically about 4 nmol/min/ml (mU/ml). Activity was tenfold lower in cultures expressing the His-tagged isomerase. The amount of soluble His-tagged LA isomerase detected on Western Blot was also tenfold lower (data not shown). This observation suggests that the presence of the C-terminal His tag interfered with the folding of the isomerase, but it did not affect enzyme catalysis. The *P. acnes* LA isomerase was expressed in yeast *Hansenula polymorpha* at activity levels comparable to that in *E. coli*. The enzyme expressed very poorly in *Bacillus licheniformis* (data not shown).

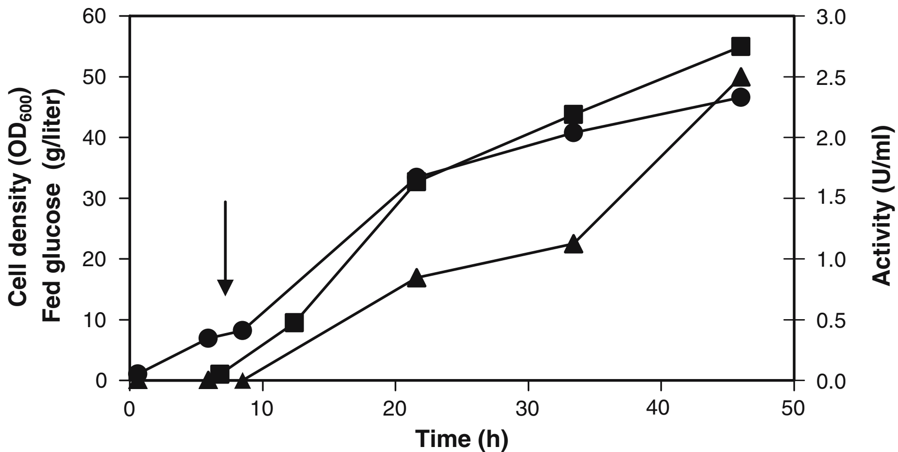

Fig. 4 Production of the recombinant LA C9 isomerase in fermentation. *E. coli* cells harboring a pET vector with the recombinant LA C9 isomerase gene were grown in a mineral salts-based medium in a 14-l fermentor. Following 6 h of growth at 37 °C, temperature was changed to 26 °C, and the culture was induced with 1 mM IPTG as indicated by the *arrow*. LA isomerase activity (*filled squares*), cell density (OD600, *filled circles*), and the amount of glucose fed (*filled triangles*) were monitored

Optimization of Isomerase Expression in Fermentation

Attempts to solubilize inclusion bodies and refold the LA isomerase using commonly used procedures did not produce active enzyme. Therefore, experiments were carried out to improve the expression of a soluble and active LA isomerase in 14-l fermentors. Cells were grown in LB broth at 37 °C for 3.5 h and induced with 0.4 mM IPTG at 26 °C. Isomerase activity reached 6.2 mU/ml after 2 h of induction and increased slightly to 7.3 mU/ml after 9 h of induction. However, activity declined to 2.1 mU/ml at 21 h post-induction. Fermentation in a mineral salt medium supplemented with 20 g/l N-Z amine and 20 g/l glucose produced tenfold higher isomerase activity. This medium was used to further test effects of IPTG concentration, induction timing, induction temperature, pH, and glucose feeding on isomerase level. Reducing the induction temperature from 37 to 26 °C led to a threefold increase in isomerase activity. Higher cell density (OD_{600}) and isomerase activity were reached in fermentation controlled at pH 7.0 than those at pH 6.5 and 7.5. Highest isomerase level (2.7 U/ml) was achieved by growing the culture at 37 °C for 6 h and inducing with 1 mM IPTG at 26 °C with glucose fed to 50 g/l (Fig. 4). Compared to fermentation in LB broth, cell density and isomerase activity increased 10- and 376-fold, respectively, under the improved conditions. However, the majority of the expressed LA isomerase protein was still in nonfunctional form in inclusion bodies. The production of active recombinant LA isomerase may be further increased by the use of a weaker promoter (such as *lac*) and additional fermentation process optimization.

Discussion

Several enzymes catalyze the formation of conjugated double bonds in long chain fatty acids. Cahoon et al. [23] cloned a diverged form of Δ^{12}-oleic acid desaturase genes (FAD2) from plants *Momordica charantia* and *Impatiens balsamia*. This enzyme forms conjugated

fatty acids by desaturation coupled with isomerization. Yeast cells expressing the FAD2 genes converted LA to α-eleostearic acid (*c*9,*t*11,*t*13, 18:3), and α-linolenic acid (*c*9,*c*12, *c*15, 18:3) to α-paranaric acid (*c*9,*t*11,*t*13,*c*15, 18:4). A polyenoic fatty acid isomerase (PFI) from *Ptilota filicina*, a red marine alga, catalyzed the isomerization of a wide range of polyunsaturated fatty acids (typically with three or more double bonds) into conjugated trienes [24, 25]. PFI had a very low level of activity to transform LA to CLA, although the structure of the conjugated product was not fully characterized. Many bacteria contain LA isomerase activity, converting LA to *c*9,*t*11 or *t*10,*c*12 CLA [13, 19, 20, 15]

P. acnes LA C9 isomerase displayed several desirable features for being developed into an enzyme catalyst. First, the *P. acnes* enzyme is a soluble protein, unlike the LA C12 isomerases from *B. fibrisolvens*, *L. reuteri*, and *C. sporogenes*, which were membrane proteins [12, 14, 16]. It is more difficult to develop a biocatalyst using a membrane protein than using a soluble protein. Secondly, although the enzyme requires FAD as a cofactor, it has no additional requirement for reducing power (NADH, NADPH) nor energy (ATP) for catalytic activity. Thirdly, the enzyme is not substrate-inhibited as is the case with the three LA C12 isomerases. However, slow growth and low LA isomerase activity are prohibitive to developing a production process using *P. acnes*. A better alternative is to clone the LA isomerase gene and to overexpress it in preferred heterologous hosts. To this end, the LA C9 isomerase was purified from *P. acnes*, and the LA isomerase gene was cloned using degenerated oligonucleotide primers designed according to the N-terminal peptide sequence of the purified enzyme. The functionality of the gene was confirmed by the synthesis of *t*10, *c*12-CLA from LA by the recombinant LA isomerase expressed in *E. coli*.

The *P. acnes* LA C9 isomerase shares little sequence homology to other enzymes except for a FAD-binding domain in the N-terminal region. The same domain is also found in *P. filicina* PFI [25], suggesting similarity in their catalytic mechanisms. Interestingly, a concentrated solution of denatured recombinant LA isomerase with a His tag had a yellowish appearance. A UV spectrum, typical of FAD-bound proteins was observed, as shown for *P. filicina* PFI [25]. This observation was the first indication that the putative FAD-binding domain in LA isomerase might actually bind FAD, although it was not known how FAD remained associated with the denatured LA isomerase that was solubilized from inclusion bodies and purified through a nickel column. Recombinant LA isomerase without His tag that was partially purified by DEAE and HIC columns also showed the yellowish color, consistent with a tightly-bound FAD cofactor. The color could serve as an indicator for chromatography fractions containing high amounts of LA isomerase. The cloned *P. acnes* LA isomerase sequence and preliminary characterizations were reported in our patent application, WO0100846 [14]. Based on this patent, Hornung et al. [26] subcloned and expressed the *lai* gene for protein structural determination. Their work confirmed the binding of FAD to the LA isomerase.

LA isomerase expressed in *E. coli* was almost totally sequestered in a nonfunctional form in inclusion bodies under the standard IPTG induction conditions. The expression of an active LA isomerase was improved 365-fold in fed batch fermentation. Several grams of active isomerase were partially purified. This has allowed experimental evaluations of the isomerase as an enzyme catalyst in CLA production (data not shown). Hornung et al. [27] also expressed the *P. acnes* isomerase gene in *Saccharomyces cerevisiae* and tobacco seeds, leading to significant increases in *t*10,*c*12 CLA content in esterified fatty acids.[1]

[1] The nucleotide sequence encoding the *P. acnes* LA C9 isomerase and its deuced amino acid sequence were deposited in Genebank with accession numbers of AX062088 and CAC25049, respectively.

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