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Controlling *cis/trans* isomerism of monounsaturated fatty acids via a recombinant cytochrome *c*-type *cis/trans* fatty acid isomerase

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Abstract

Cytochrome *c*-type *cis/trans* fatty acid isomerase (CTI) has been proposed to control *cis/trans* isomerism of unsaturated fats in lipid-related food products. A gene encoding wildtype CTI from *Pseudomonas putida* KT2440 was introduced into the pET26b/pEC86 co-expression system, and the heme C cofactor was covalently bound into the expressed CTI protein through *in vivo* cytochrome *c* maturation. The recombinant CTI, purified from *Escherichia coli* BL21(DE3), catalyzed the *cis/trans* isomerization of three edible monounsaturated fatty acids. It exhibited strong substrate selectivity for palmitoleic acid (C_{16:1}, *cis*- Δ^9), reaching an 80.93 \pm 1.78% conversion at reaction equilibrium. Notably, its promiscuity for other fatty acids (oleic acid: 29.21 \pm 5.01% and *cis*-vaccenic acid: 51.21 \pm 0.05%) was observed. Under the optimum reaction conditions (pH 7.5 and 15°C), the kinetic parameters (V_{max} , K_m , and k_{cat}) of CTI were derived as 0.035 mM \cdot min⁻¹, 0.267 mM, and 0.141 sec⁻¹, respectively, and the final catalytic efficiency (k_{cat}/K_m) was calculated as 5.26 $\times 10^2$ M⁻¹ \cdot sec⁻¹. Furthermore, structural properties of CTI were analyzed using deep learning-based protein structure prediction, suggesting the potential for specificity variability by altering loop dynamics and helix interactions surrounding the heme-binding motif. The following results would provide theoretical and practical information for CTI enzymes as novel promising industrial catalysts to control *cis/trans* isomerism of lipids in food products.

Keywords: *cis/trans* fatty acid isomerase; *cis/trans* isomerization; *Pseudomonas putida* KT2440; monounsaturated fatty acid; cytochrome *c*; pET26b/pEC86 co-expression system.

1. Introduction

In the past three decades, the consumption of industrial *trans*-fatty acids (*i.e.*, unsaturated fatty acids that contain non-conjugated carbon-carbon double bonds in *trans* configuration) has been proposed to be responsible for a higher incidence of coronary heart disease (CVD) (Kodali, 2014). Compared with *cis*-fatty acids, they have a straight aliphatic hydrocarbon chain similar to saturated fatty acids, known to increase the ratio of low-density to high-density lipoprotein cholesterol levels (LDL:HDL) in human blood (greater than that of saturated fatty acids) (Mozaffarian, Aro, & Willett, 2009). Although natural foods such as ruminant meat, dairy products, and refined oils contain *trans*-fatty acids in small amounts, the majority are industrially produced during the hydrogenation process of vegetable/fish oils for manufacturing partially hydrogenated vegetable oils (PHVOs), margarines, and shortenings (Kuhnt, Baehr, Rohrer, & Jahreis, 2011; Mozaffarian, Katan, Ascherio, Stampfer, & Willett, 2006). These detrimental lipids are found in most processed food products containing unsaturated fats because heating or frying operations can induce their production (Tsuzuki, Matsuoka, & Ushida, 2010). The World Health Organization (WHO) has declared that the elimination of *trans*-fatty acids in food products is the most effective intervention to prevent CVDs and create a healthier food supply (Ghebreyesus & Frieden, 2018). Therefore, food scientists have studied relevant scientific technologies – process improvement for hydrogenation (Iida, Takahashi, Yanagisawa, Hashimoto, & Igarashi, 2021), natural hardstock blending (Yamoneka, Malumba, Lognay, Blecker, & Danthine, 2019), trait-enhanced oils (Wilkes & Bringe, 2015), functional additives (Guo et al., 2015), chemical and enzymatic interesterification (Kim & Akoh, 2015), and other techniques (Brundiek, Evitt, Kourist, & Bornscheuer, 2012) – to reduce, remove, or replace *trans*-fatty acids in food products. Despite this, no technology has been developed that allows direct control of *cis/trans* isomerization during downstream processing of unsaturated fats.

More recently, it has been proposed that enzymatic *cis/trans* isomerization of fatty acids can be an innovative technology to manufacture ideal *trans*-free food products under mild conditions. In a bid to find novel isomerases, our research group has developed an improved method for rapid evaluation of

51 enzymatic *cis/trans* isomerization of monounsaturated fatty acids in a reversed micelle enzyme reac-
52 tion system (Park, Choi, Park, & Chang, 2023). In this previous study, an enzyme, *cis/trans* fatty acid
53 isomerase (CTI) from *Pseudomonas putida*, was initiatively suggested as a promising enzyme resource
54 to be repurposed for catalyzing the *cis/trans* isomerization of unsaturated fatty acids. A few *Pseudo-*
55 *monas* strains have evolved a unique strategy based on the *cis/trans* isomerization of membrane phos-
56 pholipids to resist external exposure to hazardous substances (Diefenbach & Keweloh, 1994; Heipieper,
57 Diefenbach, & Keweloh, 1992). Several microbiological studies revealed that CTI, which is a consti-
58 tutively expressed enzyme, rigidifies cytoplasmic membranes for rapid adaptation to external waves
59 via immediate conversion of the *cis*-fatty acid moiety of membrane phospholipids into their *trans* con-
60 figurations (Holtwick, Meinhardt, & Keweloh, 1997; Mauger, Ferreri, Chatgililoglu, & Seemann,
61 2021). This CTI enzyme is a cytochrome *c*-type hemoprotein with a heme-binding motif (CXXCH)
62 (Holtwick, Keweloh, & Meinhardt, 1999) and functions without a transient saturation of the carbon-
63 carbon double bond during the reaction (von Wallbrunn, Richnow, Neumann, Meinhardt, & Heipieper,
64 2003), indicating the direct catalysis of isomerization. These findings partially support efforts to intro-
65 duce the CTI gene into industrial bacterial strains to enhance tolerance and robustness during biocon-
66 version processes (Ahn, Lee, Bang, & Lee, 2018; Tan, Yoon, Nielsen, Shanks, & Jarboe, 2016).

67 On the other hand, there was no report to facilitate CTI enzymes for the direct *cis/trans* isomerization
68 of unsaturated fats in lipid-based food products, although microbial hemoproteins have been investi-
69 gated as major industrial catalysts that enable manufacturers to selectively produce desired compounds.
70 Only a few wildtype CTIs were isolated and characterized (Okuyama, Ueno, Enari, Morita, & Kusano,
71 1997; Pedrotta & Witholt, 1999), whereas it has been difficult to mass-produce CTI recombinant pro-
72 teins via biotechnological methodologies. It was reported that histidine-tagged CTI recombinant pro-
73 teins from *P. putida* P8 were expressed in a heterologous *Escherichia coli*-pQE60 expression system;
74 however, data on their catalytic activities are unavailable (Holtwick et al., 1999). Since cytochrome *c*-
75 type hemoproteins typically undergo cytochrome *c* maturation during membrane translocation to the

periplasm (a heme C cofactor is covalently bound to the apoprotein during this process) (Thöny-Meyer, 1997), it is highly challenging to forcibly induce expression of CTI to be in a mature form in heterologous strains without relevant functions. This led us to consider that a more advanced expression system for the production of CTI in its active form is firstly needed to evaluate its *in vitro* catalytic properties and further practical applicability as a potent controller for the *cis/trans* isomerization of unsaturated lipids. Moreover, no protein structure of CTI has been elucidated via either experimental methods or computational predictions. It is essential to investigate the protein structure and its relationship to the catalytic mechanism to further modify the enzyme tailored to the desired specifications.

In the present study, we have introduced a wildtype CTI gene from *P. putida* KT2440 into a heterologous *E. coli* BL21(DE3) based on the pET26b/pEC86 co-expression system to mass-produce CTI recombinant proteins in their active forms and consequently control the *cis/trans* isomerization of unsaturated fats directly. Catalytic properties of the purified CTI recombinant proteins against three different edible *cis*-monounsaturated fatty acids, which can be found in various food sources and products, were evaluated using the reversed micelle reaction system. In addition, the structural properties of CTI were investigated using the latest computational protein structure prediction methodologies to in-depth understand its mode of action, substrate specificity/selectivity, and potential for further adaptation. The following results would provide theoretical and practical information for introducing CTI enzymes as novel promising industrial catalysts to control *cis/trans* isomerism of lipids in food products.

2. Materials & methods

2.1. Materials

Oleic acid ($\geq 99.0\%$), elaidic acid ($\geq 98.0\%$), and kanamycin sulfate (USP standard) were purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA). *Cis*-vaccenic acid ($\geq 97.0\%$), *trans*-vaccenic acid ($\geq 99.0\%$), *cis*-11-vaccenic methyl ester ($\geq 95.0\%$), *trans*-11-vaccenic methyl ester ($\geq 95.0\%$), methyl oleate ($\geq 98.5\%$), methyl elaidate ($\geq 99.0\%$), and dioctyl sulfosuccinate sodium salt (AOT, $\geq 97.0\%$)

were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Palmitoleic acid ($\geq 98.0\%$), methyl palmitoleate ($\geq 95.0\%$), isopropyl β -D-1-thiogalactopyranoside (IPTG, $\geq 98.0\%$), and 5-aminolevulinic acid hydrochloride (ALA, $\geq 98.0\%$) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Palmitelaidic acid ($\geq 99.0\%$), methyl palmitelaidate ($\geq 98.0\%$), and chloramphenicol ($\geq 98.0\%$) were purchased from Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA). Restriction enzymes (Nco1, Nde1, and Xho1) were purchased from Takara Bio Co. (Shiga, Japan). High performance liquid chromatography-grade *n*-hexane ($\geq 95.0\%$), ethanol ($\geq 99.0\%$), and other chemicals (extra pure grade) were purchased from Daejung Chemicals & Metals Co. (Siheung, Republic of Korea).

2.2. Plasmids, strains, and media

Plasmid pET26b(+) (Novagen[®]) was used as a cloning vector, and cloning was performed through in-fusion cloning. Plasmid pEC86 (Culture Collection of Switzerland) was used for cytochrome *c* maturation (Arslan, Schulz, Zufferey, Künzler, & Thöny-Meyer, 1998). *P. putida* KT2440 strain (ATCC[®] 47054[™]), as well as its whole genome sequence, was distributed from American Type Culture Collection (Manassas, VA, USA). Heterologous *E. coli* DH5 α (Dyne Bio Co., Seongnam, Republic of Korea) and *E. coli* BL21(DE3) (SoluBL21[™], Genlantis Co., San Diego, CA, USA) strains were employed for sub-cloning of plasmid and expression of recombinant protein, respectively. All cells were propagated before use according to manufacturer instructions. The recombinant cells transformed with pET26b(+) or pEC86 plasmids were grown in Luria-Bertani (LB) media with 50 μ g/mL kanamycin or 20 μ g/mL chloramphenicol, respectively. Cells containing both pET26b(+) and pEC86 plasmids were grown in LB medium with 50 μ g/mL kanamycin and 20 μ g/mL chloramphenicol (LB_{kan/chlor}).

2.3. Construction of plasmid and dual-transformation

Genomic DNA (gDNA) of *P. putida* KT2440 strain was extracted with AccuPrep[®] Genomic DNA Extraction Kit (Bioneer Co., Daejeon, Republic of Korea). The gene encoding CTI (2,301 bp; Uniprot

ID Q88KB4) was amplified from the gDNA by overhang PCR using Pfu polymerase and two 5' overhang F/R primers (Fig. 1a). The annealing and extension temperatures of overhang PCR were set to 60°C and 72°C, respectively. The CTI DNA fragments were detected on 1%(w/v) agarose gel, purified using PureLink™ Quick Gel Extraction and PCR Purification Combo Kit (Thermo Fisher Scientific Co.), and in-fusion cloned using AccuRapid™ Cloning Kit (Bioneer Co.) into the linearized pET26b(+) vectors between restriction enzyme sites *NcoI* and *XhoI* in frame with an N-terminal pelB signal peptide (to promote periplasmic localization and maturation; MKYLLPTAAAGLLLLAAQPAMA) and a C-terminal polyhistidine fusion tag (His₆; HHHHHH) (Fig. 1b). Based on the codon usage of *E. coli* provided by the Codon Usage Database (<https://www.kazusa.or.jp/codon/>), the codon adaptation index (CAI) of the recombinant CTI gene was calculated as follows (Sharp & Li, 1987):

$$\text{Codon Adaptation Index (CAI)} = \left(\prod_{i=1}^L w_i \right)^{\frac{1}{L}}$$

where w_i and L are the codon weight for the i^{th} codon in the gene and the total number of codons in the gene, respectively. Codon weight (*i.e.*, the relative adaptiveness of a codon) is the frequency of codon usage of each codon compared to that of the optimal synonymous codon for the same amino acid. The pEC86 plasmid was also co-introduced to ensure cytochrome *c* maturation in aerobic culture conditions (Fig. 1c). The constructed pET26b(+) plasmids possessing the CTI gene were transformed into *E. coli* BL21(DE3) competent cells by heat shock (42°C, 30 sec) before making them competent cells again, and the pEC86 plasmids were subsequently transformed (Fig. 1d). Chemically competent cells were prepared using calcium chloride (CaCl₂) as previously described (Green & Rogers, 2013). *E. coli* BL21(DE3) transformants were selected by antibiotic screening and restriction enzyme treatment, and their plasmids were sequenced by Sanger sequencing (serviced by Bioneer Co.).

2.4. Expression and purification of recombinant proteins

An overnight sub-culture (7.5 mL, LB_{kan/chlor}) of dual-transformed *E. coli* BL21(DE3) cells, which

contain the constructed pET26b(+) plasmid encoding the CTI gene, as well as the pEC86 plasmid, was inoculated in a three-liter Erlenmeyer flask containing LB_{kan/chlor} broth medium (750 mL) without other carbon sources. This main culture was incubated at 37°C and 200 rpm (no humidity control) until the optical density (OD₆₀₀) reached 0.6–0.7 (mid-log phase, approximately 2–3 h), and then, the flask was placed on ice (below 4°C) for 30 min. To initiate CTI expression, IPTG and ALA were added to final concentrations of 5 µM and 500 µM, respectively, and the culture was incubated at 20°C and 200 rpm for 20 h. Cell pellets were collected by centrifugation (4,000g, 20 min, 4°C) and stored at –80°C until further use (at least 24 h). The cell pellets were resuspended in 15 mL of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM imidazole and 200 mM sodium chloride (NaCl) at room temperature, and the cells were totally lysed by sonication (8 min, 2 sec on, 2 sec off, 12 sec pause per 1 min, 30% power; NE-300Z sonicator, LabTech Co., Jeonju, Republic of Korea) on ice. Cell debris with insoluble proteins was removed by centrifugation (13,000g, 20 min, 4°C) and supernatant with soluble proteins was obtained as cleared lysate (approximately 20 mL/batch). The cleared lysate was sterile-filtered through a 0.45 µm cellulose acetate filter and resolved using an ÄKTA goTM fast protein liquid chromatography (FPLC) system (Cytiva Co., Marlborough, MA, USA) equipped with a 1 mL Ni-NTA affinity column (HisTrapTM High Performance; Cytiva Co.) and ultraviolet (UV) detector. His-tagged CTI recombinant proteins were eluted by running a linear imidazole gradient (10 to 500 mM) over 15 column volumes (1 mL/min, 0.5 mL fraction). The purity of each fraction was analyzed by SDS-PAGE analysis, and then, pure fractions were collected, dialyzed overnight in 50 mM potassium phosphate buffer (pH 7.5) using a 10 kDa molecular weight cut-off (MWCO) dialysis sack (Thermo Fisher Scientific Co.), and concentrated via a 50 kDa MWCO centrifugal filter (Sigma-Aldrich Co.). Protein concentration was measured by the Bradford assay using bovine serum albumin as the standard protein.

2.5. CTI activity assay

The CTI activity against monounsaturated fatty acids was determined in a reversed micelle reaction

175 system, as previously described (Park et al., 2023). In detail, 0.09 mL of the purified CTI dissolved in
 176 50 mM potassium phosphate buffer (pH 7.5) was added to 4.41 mL of 113.38 mM AOT/isooctane (10
 177 of *R*-value) before vigorous vortexing for 1 min to produce stable reversed micelles (reaction medium).
 178 The reaction medium with a magnetic stirrer was pre-incubated for 10 min at 20°C and 800 rpm, and
 179 the reaction was initiated by adding 0.5 mL of substrate dissolved in isooctane. After the CTI reaction,
 180 an aliquot of the reactant (300 µL) was sampled, and then, isooctane was totally removed by evapora-
 181 tion at room temperature using a nitrogen evaporator (MGS-3100; Eyela Co., Bohemia, NY, USA).
 182 Fatty acids in the dried reactants were methylated using a Fatty Acid Methylation Kit (Nacalai Tesque
 183 Co., Kyoto, Japan) according to manufacturer instructions. The dried samples were sufficiently dis-
 184 solved with 0.5 mL of methylation reagent A (toluene:methanol=52:48, v/v) and 0.5 mL of methyla-
 185 tion reagent C (strong acidic BF₃-methanol solution) before incubation for 20 min at 37°C. Generated
 186 fatty acid methyl esters (FAMES) were fractionated with *n*-hexane (1 mL), followed by washing with
 187 deionized water (1 mL). The washed samples were filtered through a 0.45 µm PTFE filter and analyzed
 188 through a YL6500 gas chromatography (GC) system (Young In Chromass Co., Anyang, Republic of
 189 Korea) equipped with a cyanopropyl phase DB-FastFAME capillary column (30 m × 0.25 mm × 0.25
 190 µm; Agilent Technologies Co., Santa Clara, CA, USA) and a flame ionization detector (FID). Helium
 191 (≥99.999%) was used as a carrier gas at a flow rate of 1.0 mL/min (constant flow), and other details of
 192 GC-FID analysis are summarized in Supplementary Data Table S1. Calibration curves for methyl ole-
 193 ate, methyl elaidate, methyl *cis*-vaccenate, and methyl *trans*-vaccenate were adapted from our previous
 194 study (Park et al., 2023). Calibration curves for methyl palmitoleate and palmitelaidate, together with
 195 resolution factor, were newly derived in the present study (Supplementary Data Fig. S2). The separa-
 196 tion of adjacent peaks was characterized by resolution factor (R_s) as follows:

$$\text{Resolution factor } (R_s) = 1.18 \times \left[\frac{t_{R2} - t_{R1}}{W_{h2} + W_{h1}} \right]$$

198 where t_R is the retention time for each peak, W_h is the full width at half maximum of each peak, and an

indexed number (1 and 2) refers to the former and the latter of adjacent peaks, respectively. The catalytic activity of CTI was assessed by calculating the conversion rate (%) of *cis* configuration to *trans* configuration as follows:

$$\text{Conversion rate (\%)} = \frac{[trans]_t}{[cis]_t + [trans]_t} \times 100$$

where $[cis]_t$ and $[trans]_t$ are the amounts of fatty acid in *cis* and *trans* configuration at specific reaction time t , respectively. For the kinetic analysis, the reaction under given conditions was analyzed based on the Michaelis-Menten assumption using SigmaPlot software (ver. 12.5; Systat Software Co., San Jose, CA, USA). The Michaelis-Menten assumption was validated by Hanes-Woolf double-reciprocal plotting as follows:

$$\frac{[S]}{v} = \frac{1}{V_{max}} \times [S] + \frac{K_m}{V_{max}}$$

where $[S]$ is the concentration of substrate, v is the initial velocity of the reaction, V_{max} is the maximum initial velocity of the reaction, and K_m is the dissociation constant of the enzyme-substrate complex.

2.6. Genome-based computational analysis of protein structure

Both an original CTI gene (Uniprot ID Q88KB4) encoded in the gDNA of *P. putida* KT2440 and a recombinant CTI gene encoded in the constructed pET26b(+) plasmid (Supplementary Data Fig. S3) were used for computational protein structure prediction. Signal peptide type, subregions, and cleavage site were predicted from the genes using SignalP 6.0 software (<https://services.healthtech.dtu.dk/services/SignalP-6.0/>), which is based on a transformer protein language model trained on a massive dataset of unlabeled amino acid sequences (Teufel et al., 2022). The likelihoods of signal peptide at each amino acid residue were expressed as region probabilities (P), and cleavage sites were predicted in the correct signal peptide type. Three-dimensional (3D) model for a target protein was predicted from the gene through deep learning-based RoseTTAFold software (<https://robetta.bakerlab.org/>), which is a

prediction architecture with a three-track deep neural network to acquire relationships within and between amino acid sequences, distances, and coordinates in the protein structure simultaneously (Baek et al., 2021). Angstrom error estimates of amino acid residues in the predicted model were calculated using a deep learning framework (DeepAccNet) to guide protein structure refinement, and the confidence value (accuracy; 1.0 good, 0.0 bad) of the prediction was derived from the local distance difference test (lDDT) score (Hiranuma, Park, Baek, Anishchenko, Dauparas, & Baker, 2021). The final 3D structure model of protein and ligand was rendered using PyMOL software (<https://pymol.org/2/>) and UCSF Chimera software (<https://www.cgl.ucsf.edu/chimera/>) (Pettersen et al., 2004).

230

3. Results & discussion

3.1. Molecular cloning of CTI gene in the pET26b/pEC86 co-expression system

Cytochrome *c*-type CTIs are occasionally discovered in small amounts in Gram-negative bacteria (mainly *Pseudomonas* and *Vibrio* species), and these enzymes are constitutively expressed to ensure their high adaptability in harsh environments (Eberlein, Baumgarten, Starke, & Heipieper, 2018). We selected *P. putida* KT2440 as a source of genes because its catalytic activity in the form of a periplasmic extract was putatively identified against monounsaturated fatty acids in a previous study, and its complete genome sequence was revealed (Nelson et al., 2002; Park et al., 2023). One copy of the gDNA of *P. putida* KT2440 (ATCC[®] 47054[™]) strain consists of 6,185,012 base pairs and 5,650 coding sequences, and a putative gene encoding CTI (Uniprot ID Q88KB4) corresponds to 2,708,078–2,710,378 base pairs (2,301 bp/766 amino acids, Supplementary Data Fig. S4a). A BLAST analysis, using this CTI gene from *P. putida* KT2440 as a reference, revealed that the majority of putative CTI genes in *Pseudomonas* strains are highly conserved, with an average sequence identity of 90% (data not shown). Computational prediction of the protein structure based on the nucleotide sequence found two important structural features of the putative CTI protein from *P. putida* KT2440 (Supplementary Data Fig. S4b). One is an N-terminal signal peptide (MVHRILAGAFAKKOSGAVFG, 1–20 amino

acid residues), and the other is an iron-containing heme-binding motif (CVACH, 43–47 amino acid residues) right after 23 amino acid residues from the signal peptide. These results indicate that expressed CTI proteins are localized in the periplasm and undergo post-translational cytochrome *c* maturation during translocation. Indeed, the catalytic activities of all previously reported CTIs from *Pseudomonas* strains were found in their periplasmic fraction (Okuyama et al., 1997; Park et al., 2023; Pedrotta et al., 1999).

Gram-negative bacteria possess a distinct space between the inner cytoplasmic membrane and the outer membrane, known as the periplasm. Most protective enzymes, constitutively expressed in Gram-negative bacteria, are typically located in the periplasm because they are needed to respond to immediate changes in the external environment. These enzymes are initially produced within the cytoplasm in unfolded forms, and signal peptides at their N-terminals are then recognized by the Sec-dependent secretory system for translocation across the cytoplasmic membrane (Owji, Nezafat, Negahdaripour, Hajiebrahimi, & Ghasemi, 2018). Computational prediction of the signal peptide in the wildtype CTI from *P. putida* KT2440 revealed the presence of a signal peptide at its N-terminus, which is expected to be recognized by the Sec pathway and cleaved by a signal peptidase I (Sec/SP1-type) (Supplementary Data Fig. S4c). The cleavage site of the signal peptide was predicted to be between the 20th and 21st amino acid residues, and the molecular weight of the wildtype CTI after cytochrome *c* maturation is calculated as approximately 85 kDa. This native signal peptide is known to work similarly in other Gram-negative bacteria, such as *E. coli* (Tan et al., 2016); however, substituting it with a *pelB* signal peptide (MKYLLPTAAAGLLLLAAQPAMA) can significantly enhance the periplasmic localization of CTI proteins in heterologous *E. coli* BL21(DE3), due to the structural differences in their subregions (n-, h-, and c-region) that are responsible for translocation capacity and efficiency (Owji et al., 2018). Therefore, a pET26b(+) plasmid, encoding a *pelB* signal sequence and a polyhistidine fusion tag upstream and downstream of the multiple cloning site, respectively, was selected as an expression vector for the cloning of the putative CTI gene from *P. putida* KT2440 in *E. coli* (Fig. 1b).

272 Furthermore, the wildtype CTI from *P. putida* KT2440 is identified as a cytochrome *c*-type protein
273 because of its putative heme-binding motif. Cytochrome *c* is a metalloprotein that contains a covalently
274 bound heme as a prosthetic group, which is capable of undergoing oxidation and reduction (or binding
275 with other compounds) as its iron converts between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms (Thöny-
276 Meyer, 1997). In bacteria, heme attachment to expressed apocytochrome polypeptides by two thioether
277 bonds occurs in the periplasm by a specific membrane protein complex after their translocation across
278 the cytoplasmic membrane (Thöny-Meyer, 1997). This post-translational modification of proteins is
279 called cytochrome *c* maturation, and CTI proteins must covalently bind with a heme cofactor at their
280 heme-binding motifs (CXXCH) to catalyze the *cis/trans* isomerization of unsaturated fats. Unfortu-
281 nately, cytochrome *c* maturation genes (*ccmABCDEFGH*) are endogenously present on the chromo-
282 some of *E. coli* and function under anaerobic respiratory conditions (Thöny-Meyer, Fischer, Künzler,
283 Ritz, & Hennecke, 1995). To overcome this limitation, a pEC86 plasmid, encoding the relevant genes
284 constitutively expressed from the *tet* promoter (Arslan et al., 1998), was adopted as part of the co-
285 expression system (Fig. 1c). The pEC86 plasmid has been used to complement heterologous expres-
286 sion of recombinant cells harboring cytochrome *c*-type proteins under aerobic respiratory culture con-
287 ditions to enzymatically synthesize enantiomeric compounds (Kan, Huang, Gumulya, Chen, & Arnold,
288 2017). Considering these structural features, the wildtype CTI gene from *P. putida* KT2440 was in-
289 fusion cloned into *E. coli* BL21(DE3) based on the pET26b/pEC86 co-expression system (Fig. 1d).

290 Under annealing temperature gradient conditions, overhang PCR was performed to amplify a single
291 CTI fragment (2,274 bp) with the desired 5' overhang from the gDNA of *P. putida* KT2440 (Fig. 2a).
292 A clear single DNA band for amplified CTI DNA fragments was detected between 2.0 kb and 3.0 kb
293 under all temperature conditions, and that of 60.0°C without unspecific DNA bands and smearing was
294 further purified. The purified DNA fragments were in-fusion cloned into linearized pET26b(+) vectors
295 (5,360 bp), in frame with an N-terminal *pelB* signal peptide and a C-terminal polyhistidine fusion tag,

296 resulting in pET26b(+)-CTI plasmids (7,538 bp) (Fig. 2b). Notably, the pET26b(+) vectors were line-
297 arized through two restriction enzymes, *NcoI* (CCATGG) and *XhoI* (CTCGAG), and after in-fusion
298 cloning, the restriction site of *NcoI* disappeared in the pET26b(+)-CTI plasmids (Supplementary Data
299 Fig. S3). Instead, the restriction enzyme site of *NdeI* (CATATG) at nucleotide position -3 to +2 of the
300 translational start codon (ATG) of the open reading frame was used to confirm the insertion of a CTI
301 gene. Two plasmids, pET26b(+)-CTI and pEC86, were transformed into *E. coli* BL21(DE3) competent
302 cells, and the transformants were screened for antibiotic resistance. Dual-transformants possessing
303 both plasmids were confirmed by gel electrophoresis (Fig. 2c) and sequencing. In addition, each plas-
304 mid is recommended to be individually transformed because it was empirically challenging to simul-
305 taneously transform both pET26b(+)-CTI and pEC86 plasmids into competent cells.

306

307 3.2. Heterologous expression of CTI recombinant protein

308 Recombinant CTIs with an N-terminal pelB signal peptide and a C-terminal polyhistidine fusion tag
309 (Fig. 3a) were expressed in the pET26b/pEC86 co-expression system under IPTG induction and ALA
310 supplement, and their mature proteins were purified in soluble form by affinity chromatography. First
311 of all, according to computational prediction, the recombinant CTI, which carries a pelB signal peptide,
312 is capable of being recognized by the Sec pathway and translocated into the periplasm of Gram-nega-
313 tive bacteria (Fig. 3b), as intended. The cleavage site of the signal peptide was predicted to be between
314 the 22nd and 23rd amino acid residues, and consequently, the molecular weight of the recombinant CTI
315 after cytochrome *c* maturation is calculated as approximately 86 kDa, which is slightly higher than that
316 of the wildtype CTI, due to the attached polyhistidine fusion tag. However, although the signal peptide
317 of the recombinant CTI was adjusted, and its codon bias (0.75 of CAI value) was low enough to allow
318 heterologous expression in *E. coli* (Supplementary Data Fig. S5), the CTI proteins were obtained in an
319 insoluble form when expressed without dual-transformation of a pEC86 plasmid. Only a few soluble
320 CTI proteins were purified using solubilizing buffers containing 1%(w/v) cationic CHAPS detergents,

and the purified CTI proteins had an incorrect size (below 75 kDa) and exhibited no catalytic activity (Supplementary Data Fig. S6). It was probably due to the lack of cytochrome *c* maturation in CTI.

Cytochrome *c*-type hemoproteins need post-translational maturation in which the heme C cofactor is covalently bound to the heme-binding motif. Correct incorporation of heme molecules into recombinant cytochrome *c*-type apoprotein is essential for the solubility and catalytic activity of its holoprotein in heterologous expression (Arslan et al., 1998; Varnado & Goodwin, 2004). Furthermore, a sufficient supply of heme precursor (ALA) is crucial for the overexpression of recombinant hemoproteins because a shortage of heme to be conjugated with overexpressed hemoproteins leads to the production of immature proteins (Ramzi, Hyeon, & Han, 2015), which can destabilize proteins and cause inclusion bodies or reduced catalytic activity. The pET26b/pEC86 co-expression system with the ALA supplement successfully mass-produced recombinant CTIs in mature form, and consequently, their stability and solubility dramatically increased. His-tagged recombinant CTIs were purified from cleared lysates of the transformant at 227.14–277.80 mM imidazole (28–30 fractions, 0.5 mL/fraction) in Ni-NTA affinity chromatography (Fig. 3c–d). The detected molecular weights of eluted proteins in those pure fractions were between 75 kDa and 100 kDa (Fig. 3e), which corresponds to that of the recombinant CTI (approximately 86 kDa). Several small protein bands (similar to those of incorrect recombinant proteins from the single pET26b(+) expression system) were detected, indicating that unknown destabilized proteins were partially produced during overexpression. Of course, the recombinant CTI proteins were completely purified after chromatographic resolution, as well as dialysis and concentration. These results suggest that the pET26b/pEC86 co-expression system is highly suitable for the heterologous expression of cytochrome *c*-type CTI from *P. putida* KT2440 in *E. coli*.

3.3. Catalytic properties of CTI against monounsaturated fatty acids

The original catalytic activity of CTI in the bacterial periplasm is known for converting *cis*-unsaturated fatty acid moieties of membrane phospholipids into their *trans* configurations (Holtwick et al.,

1999). On the other hand, wildtype CTIs purified from *Pseudomonas* showed catalytic activity on monounsaturated fatty acids, especially palmitoleic acid, under *in vitro* reaction conditions (Okuyama et al., 1997; Pedrotta et al., 1999). Based on these findings, the catalytic activity of the purified recombinant CTI against three edible monounsaturated fatty acids in *cis* configuration – oleic acid (C_{18:1}, *cis*-Δ⁹), *cis*-vaccenic acid (C_{18:1}, *cis*-Δ¹¹), and palmitoleic acid (C_{16:1}, *cis*-Δ⁹) – was evaluated by conducting the reaction under the reversed micelle reaction system and tracking the formation of *trans*-fatty acids over reaction time until reaching reaction equilibration (Fig. 4a). The GC-FID peaks corresponding to their FAMES in both *cis* and *trans* configurations were simultaneously observed in the GC-FID chromatograms at the early stage of the reaction (Supplementary Data Fig. S7). The catalytic activity of the wildtype CTI from *P. putida* KT2440, which was detected in the periplasmic extract (Park et al., 2023), was maintained after molecular cloning and heterologous expression in *E. coli*. Notably, the recombinant CTI showed strong substrate selectivity towards palmitoleic acid, similar to the previous results, although it exhibited unique promiscuous substrate specificity for other monounsaturated fatty acids. The final conversion rate (%) of oleic acid, *cis*-vaccenic acid, and palmitoleic acid at reaction equilibration was calculated as 29.21±5.01%, 51.21±0.05%, and 80.93±1.78%, respectively (Fig. 4b). Consequently, the most efficient substrate for the recombinant CTI was palmitoleic acid, which is the major unsaturated fatty acid moiety of membrane phospholipids in *P. putida* KT2440 (Muñoz-Rojas, Bernal, Duque, Godoy, Segura, & Ramos, 2006). These results indicate that CTI catalysis under purified state only favors the formation of *trans* isomers, reminiscent of its original activity in the periplasm (Fischer, Schauer, & Heipieper, 2010), and its protein structure around the heme-binding motif (active site) may be oriented towards its original targets, palmitoleic acid or *cis*-vaccenic acid, over oleic acid (rarely found in bacteria). Nevertheless, the recombinant CTI may have a more relaxed or larger hydrophobic pocket than other wildtype CTIs, considering its promiscuous substrate specificity for unsaturated fatty acids with different alkyl chain lengths or double bond positions. This promiscuity can be exploited for controlling *cis/trans* isomerism of unsaturated fats in lipid-related food products.

Moreover, the effects of physicochemical conditions (pH and temperature) on the catalytic activity of the recombinant CTI from *P. putida* KT2440 were investigated to determine its optimum reaction conditions against its major substrate, palmitoleic acid (Fig. 5). First, at the same reaction temperature (20°C), the catalytic activity of the recombinant CTI exhibited a typical bell-shaped curve with a broad peak occurring around pH 5.0–9.0 (Fig. 5a). The relative activity of CTI peaked at pH 7.5 and declined sharply when the pH deviated from that value, resulting in a 70% reduction in activity at either pH 5.0 or pH 9.0. It was highly similar to the behavior of wildtype CTIs from different *Pseudomonas* strains (Okuyama et al., 1997; Pedrotta et al., 1999). These results indicate that CTI is optimized for the conditions of the bacterial periplasm, which maintains a pH value around 7.0–8.0 (Wilks & Slonczewski, 2007), and also this neutral pH range corresponds to the optimal growth condition for *Pseudomonas* bacteria. Compared with this, which is influenced by amino acid composition (mainly side chains), the recombinant CTI exhibited a unique catalytic response depending on the reaction temperature (Fig. 5b). Most bacterial enzymes, including the wildtype CTIs (Okuyama et al., 1997; Pedrotta et al., 1999), have their optimum reaction temperature in the range of 25–45°C, which is ideal for mesophilic bacterial growth. However, the highest catalytic activity of the recombinant CTI from mesophilic *P. putida* KT2440 was observed at 15°C and retained approximately 90% of its activity at relatively low reaction temperatures (5–10°C). It showed a significant decline above 20°C, being experimentally undetectable beyond 50°C. This peculiar property of the recombinant CTI was likely due to the presence of a C-terminal polyhistidine fusion tag and induced expression in a heterologous strain at low temperature, which could result in a slight discrepancy in protein folding and, consequently, structural instability of the cytochrome *c* domain. Of course, it is necessary to compare it with the wildtype CTI purified from *P. putida* KT2440 or the recombinant CTI protein without any fusion tag or additional amino acids.

Finally, under optimum reaction conditions (pH 7.5 and 15°C), the exploratory kinetic parameters of the recombinant CTI from *P. putida* KT2440 against palmitoleic acid were determined (Fig. 6). The

initial velocity at each palmitoleic acid concentration (0.10–1.20 mM) was derived from a linear regression of the time-course CTI reaction (0–30 min). The CTI reaction was dependent on the substrate concentration and followed a typical Michaelis-Menten enzyme kinetic model (Fig. 6a), as expected in our previous study (Park et al., 2023). The values of V_{max} and K_m were determined as $0.035 \text{ mM} \cdot \text{min}^{-1}$ and 0.267 mM based on Hanes-Woolf double-reciprocal plotting (Fig. 6b). From these kinetic parameters, the values of turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were calculated as 0.141 sec^{-1} and $5.26 \times 10^2 \text{ M}^{-1} \cdot \text{sec}^{-1}$, respectively. Notably, the recombinant CTI exhibited stronger activity than the periplasmic extract containing the same enzyme, considering the differences in enzyme purity and substrate (palmitoleic acid versus oleic acid). One previous study on CTI purified from *Pseudomonas* sp. strain E-3 determined the kinetic parameters using palmitoleic acid as a substrate (Okuyama et al., 1997). The substrate affinity ($1/K_m$) of this wildtype CTI (8.50 mM^{-1}) appears to be relatively higher than our recombinant CTI (3.75 mM^{-1}) in magnitude, whereas its unique substrate promiscuity revealed in the present study is considered to be a superior property for controlling *cis/trans* isomerism of unsaturated fats in lipid-related food products. Therefore, in future studies, it should be investigated whether the enzyme kinetic properties of the recombinant CTI are actually dissimilar between various kinds of unsaturated fatty acids and acylglycerol species.

3.4. Prediction of CTI protein structure and its structural characteristics

To understand catalytic characteristics and develop biotechnologically engineered CTI enzymes for practical application by rational design or directed evolution, it is imperative to elucidate their protein structures. Unfortunately, there is currently no protein structure information available in the Protein Data Bank for CTIs and their protein homologs. For this reason, in the present study, a genome-based protein 3D structure for the recombinant CTI from *P. putida* KT2440 was proposed by RoseTTAFold structure prediction (Fig. 7). Based on its amino acid sequence, RoseTTAFold predicted various mod-

419 els for protein 3D structures with atomic precision and calculated the probability density for the dis-
420 tance (Å) between each amino acid residue pair (Baek et al., 2021). We removed an N-terminal pelB
421 signal peptide region (1–22 amino acid residues) up to the putative cleavage site to predict the tertiary
422 protein structure of the recombinant CTI in mature form. After RoseTTAFold prediction, the heme C
423 cofactor with an iron ion as the central atom was computationally aligned in close proximity to the
424 heme-binding motif (active site) of the apoprotein. The estimated confidence value for the best protein
425 structure model was calculated as 0.80, which indicates that the model is of high accuracy. The ang-
426 strom error estimates for each amino acid residue of the best model ranged from 0.64 to 10.56 Å, while
427 amino acid residues except the N-terminus (signal peptide-cleaved pro-region) and the C-terminus
428 (polyhistidine fusion tag) were below 4.00 Å (Supplementary Data Fig. S8).

429 The truncated N-terminal loop region (23–32 amino acid residues after the cleavage site), containing
430 hydrophobic amino acids (hydrophobic:neutral=6:4), was predicted with slight imprecision since pre-
431 dicting the folding state of the pro-region after cleavage and translocation by the Sec-dependent secre-
432 tory pathway still remains a challenge. In contrast, the cytochrome *c* domain region (comprising amino
433 acid residues 33–190) consisting of six short α -helices, several extra loops, and a heme-binding motif
434 (CVACH, amino acid residues 46–50), was predicted to be well-folded in state, probably due to its
435 high structural homology with other cytochrome hemoproteins from varied origins. The formation of
436 cytochrome *c*-type hemoproteins involves two thioether bonds between the two vinyl groups of heme
437 and two cysteine thiol groups in the heme-binding motif of the apoprotein (Thöny-Meyer, 1997).
438 Therefore, to ensure proper protein folding into the final tertiary structure, the heme C cofactor must
439 be covalently bound to the two thiol groups of cysteines (Cys46 and Cys49) in the heme-binding motif
440 of the recombinant CTI through post-translational cytochrome *c* maturation. The central iron ion (Fe^{2+})
441 of heme is axially coordinated to the proximal side chain of a histidine ligand (His50), which is also
442 strictly conserved in the heme-binding motif. The other axial coordination site of heme remains to be

443 coordinated by distal substrate ligands, in the case of CTI, the carbon-carbon double bond in unsatu-
444 rated fatty acids or phospholipids. Notably, loop dynamics and helix interactions around the heme-
445 binding motif (active site) can determine the substrate binding properties of CTI, and it may be con-
446 served to favor its original substrates, especially palmitoleic acid. Nevertheless, the size of the binding
447 pocket and substrate tunnel in the predicted CTI model (Fig. 7, bottom view) is large to accommodate
448 a wide range of unsaturated fatty acids and acylglycerol species, determining its promiscuous substrate
449 specificity. Moreover, this predicted CTI structure suggests that its substrate specificity and selectivity
450 can be artificially altered and reinforced through directed evolution or rational protein engineering. Of
451 course, the actual shape and molecular dynamics of this space should be further elucidated by experi-
452 mental determination like X-ray crystallography for a more in-depth investigation of this.

453 In Gram-negative bacteria, the cytochrome *c* maturation of hemoproteins is tightly linked to the Sec
454 pathway and occurs in concert with membrane translocation of apoprotein (Sec/SP1), membrane trans-
455 location of heme (ccmA–D), and shuttling/attachment of heme to the apoprotein (ccmE–G) (Stevens,
456 Mavridou, Hamer, Kritsiligkou, Goddard, & Ferguson, 2011). A heme-binding motif oxidizes to form
457 disulfides that are reduced by ccmG, and the apoprotein and a heme cofactor are coupled by the heme
458 lyase ccmF before ccmH recognizes CXXCH motifs to receive heme from ccmE and covalently attach
459 it to the heme-binding motif (Brausemann, Zhang, Ilcu, & Einsle, 2021). During these comprehensive
460 processes, protein folding of hemoproteins occurs without any known involvement of other chaperones
461 (Thöny-Meyer, 1997), and in other words, the tertiary structure of the cytochrome *c* domain depends
462 on its amino acid sequence. This process demonstrates that the recombinant CTI, when heterologously
463 expressed in *E. coli*, can exhibit substrate specificity similar to that of the wildtype CTI. It also suggests
464 that it is possible to modulate the molecular dynamics of loop and helix structures around the heme-
465 binding motif through genetic mutations of specific amino acid residues. In addition, the CTI from *P.*
466 *putida* KT2440 has a larger structure than other cytochrome *c* hemoproteins (usually 10–20 kDa). The

roles of other protein domains (191–760 amino acid residues) are still unknown, and homology structure alignments failed to identify similar structures with high identity (above 30%). These three individual domains are structurally similar (Fig. 7, side view), containing one β -sheet and several α -helices, and the cytochrome *c* domain is completely covered with these bulky domains like a sunshade parasol (Fig. 7, top view). Not fully understood, it was theoretically proposed that CTI activity in the periplasm is regulated by enabling the enzyme to access phospholipids in the cytoplasmic membrane (Heipieper et al., 2001). A catalytic cytochrome *c* domain of CTI may be only accessible to the target if cytoplasmic membranes are disintegrated by external stresses that increase membrane fluidity. The bulky domain in the predicted model may hinder the enzyme from fully embedding or penetrating the cytoplasmic membrane, resulting in it being partially intercalated into the lipid bilayer at the appropriate depth for the *cis/trans* isomerization of fatty acid moieties. These structural properties of CTI will be invaluable when applied to lipid-related food products containing oil droplets or bulky oils, as the hydrophilic enzyme faces challenges accessing the substrate in such contexts.

4. Conclusions

Using food lipid compounds as reactants, such as edible oils and fats, microorganisms are practically limited, and a single enzyme needs to be employed, either in free or immobilized form. More recently, a cytochrome *c*-type CTI from Gram-negative bacteria has been proposed as a promising enzyme to facilitate the direct control of *cis/trans* fatty acid isomerization in lipid-related food products without saturation, hydrolysis, and other additional processes. In the present study, a heterologous expression platform for CTI from *P. putida* KT2440 was established based on the pET26b/pEC86 co-expression system. The recombinant CTI was successfully expressed in *E. coli* BL21(DE3) and purified in highly soluble and active form by Ni-NTA affinity chromatography. Then, its catalytic activity on three different monounsaturated fatty acids was evaluated in a reversed micelle reaction system and revealed

its unique promiscuous substrate specificity. Furthermore, computational prediction of the protein tertiary structure for the recombinant CTI was performed to demonstrate its folding and enzymatic nature, forming the basis for biotechnological manipulation of its synthetic capabilities. These results provide theoretical information for investigating CTI enzymes in biotechnological processes, and hopefully, the *cis/trans* isomerization of unsaturated lipids will be added to the extensive processing repertoire of the food and lipid industry. Of course, at present, recombinant CTI with robustness and promiscuity can serve as an enzyme resource for manufacturing both *trans*-fatty acids and *cis/trans* isomer mixtures under mild conditions in the various fields because CTI catalysis favors the formation of *trans* isomers.

CRedit Authorship Contribution Statement

Jun-Young Park: Conceptualization, Investigation, Data curation, Writing - Original draft, Writing - Review & Editing, Visualization. **Yun-Seo Jung:** Investigation, Validation, Formal analysis, Writing - Review & Editing. **Dimitris Charalampopoulos:** Writing - Review & Editing. **Kyung-Min Park:** Writing - Review & Editing, Project administration, Funding acquisition. **Pahn-Shick Chang:** Conceptualization, Writing - Review & Editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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516

517 **Data Availability**

518 Data will be made available on request.

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Figure captions

Fig. 1. Overview of cloning strategy for *cis/trans* fatty acid isomerase (CTI) from *Pseudomonas putida* KT2440. (a) Primers used for overhang PCR amplification of CTI fragments from the genomic DNA (gDNA) of *P. putida* KT2440. (b) In-fusion cloning of CTI fragments with overhangs into pET26b(+) vectors linearized by two restriction enzymes (Nco1 and Xho1). (c) Genetic map of two plasmids used in this study. The CTI gene encoded in the constructed pET26b(+) plasmid can be expressed by IPTG induction, and CTI proteins are subsequently matured by the cytochrome *c* maturation system, which is constitutively expressed by pEC86 plasmid. (d) Schematic diagram for dual-transformation of the plasmids into *Escherichia coli* BL21(DE3) for heterologous expression of CTI proteins.

Fig. 2. Molecular cloning of the *cis/trans* fatty acid isomerase (CTI) gene from *Pseudomonas putida* KT2440. (a) Gradient PCR amplification of CTI fragments with overhangs. The DNA bands expected as amplified CTI genes (2,274 bp) were detected between 2.0 kb and 3.0 kb. The annealing temperature range was from 55.0°C to 60.0°C, and 60.0°C was chosen (no detection of unspecific bands). M, DNA molecular marker. (b) In-fusion cloning of the CTI fragments into pET26b(+) plasmids. Lane 1, linearized pET26b(+) plasmid (5,360 bp, Xho1); Lane 2, linearized pET26b(+) plasmid with the CTI fragments (7,538 bp, Xho1); Lane 3, linearized pET26b(+) plasmid with the CTI fragments (7,538 bp, Nde1, Xho1). (c) Dual-transformation of pET26b(+) plasmid with the CTI fragments (7,538 bp) and pEC86 (11,753 bp) plasmid into *Escherichia coli* BL21(DE3). Lane 1, plasmids purified from *E. coli* BL21(DE3) dual-transformants and linearized by restriction enzymes (Nco1, Xho1).

Fig. 3. Heterologous expression and purification of *cis/trans* fatty acid isomerase (CTI) from *Pseudomonas putida* KT2440. (a) Predicted structural features of the putative CTI recombinant protein. The

original N-terminal signal peptide of CTI was replaced with the pelB signal peptide, and the polyhistidine tag (His₆) for purification was attached to the C-terminus. (b) Computational prediction of signal peptide and cleavage site (CS, dash dot line) of the putative CTI recombinant protein. The subregion probability of n-region (circle), h-region (square), c-region (diamond), and non-signal peptide region (triangle) at each amino acid position was predicted. Sec/SP1, the secretory signal peptides transported by the Sec translocon and cleaved by signal peptidase I. (c, d) Purification of his-tagged CTI proteins by a FPLC equipped with an Ni-NTA affinity column. Cleared lysates extracted from transformants were resolved with a linear imidazole gradient (10–500 mM). (e) SDS-PAGE analysis of eluted proteins in each fraction. The protein bands expected as CTI recombinant proteins (approximately 86 kDa) were detected between 75 kDa and 100 kDa. M, protein molecular marker; Lane 1, total cell proteins; Lane 2, insoluble proteins; Lane 3, cleared lysate; Lane 4, unbound fraction (integrated 3–12 fractions, 1.0 mL/fraction); Lane 5–16, imidazole-eluted fractions (26–37 fractions, 0.5 mL/fraction); Lane 17, purified his-tagged CTI proteins (integrated 28–30 pure fractions) after dialysis and concentration.

Fig. 4. Control of the *cis/trans* isomerism of unsaturated fatty acids by the *cis/trans* fatty acid isomerase (CTI) recombinant protein. (a) Time-course of the production of *trans*-monounsaturated fatty acids from each *cis*-monounsaturated fatty acid (substrate) until reaction equilibration. EA, elaidic acid (C_{18:1}, *trans*-Δ⁹); VA, vaccenic acid (C_{18:1}, *trans*-Δ¹¹); PEA, palmitelaidic acid (C_{16:1}, *trans*-Δ⁹). (b) Conversion rate (%) of *cis* configuration to *trans* configuration after the CTI reaction. Asterisk denotes significant differences between the data ($p < 0.05$, Duncan's multiple range test). OA, oleic acid (C_{18:1}, *cis*-Δ⁹); cVA, *cis*-vaccenic acid (C_{18:1}, *cis*-Δ¹¹); POA, palmitoleic acid (C_{16:1}, *cis*-Δ⁹).

Fig. 5. Optimum reaction conditions for the *cis/trans* fatty acid isomerase (CTI) recombinant protein against palmitoleic acid (C_{16:1}, *cis*-Δ⁹). (a) Effects of reaction pH (2.0–11.0) on the reaction. Different

buffers were used to control each range of reaction pH condition. Sodium acetate (circle, pH 2.0–6.0); Potassium phosphate (square, pH 6.0–8.0); Tris-HCl (diamond, pH 8.0–9.0); Glycine-NaOH (triangle, pH 9.0–11.0). (b) Effects of reaction temperature (5–60°C) on the reaction. All reactions were conducted at the optimal pH condition. Asterisk denotes significant differences between the data ($p < 0.05$, Duncan's multiple range test).

Fig. 6. Enzyme kinetic analysis of the *cis/trans* isomerization of palmitoleic acid ($C_{16:1}$, *cis*- Δ^9) by the *cis/trans* fatty acid isomerase (CTI) recombinant protein. (a) Michaelis-Menten plot of the CTI reaction. The initial velocity (v) at each substrate concentration (0.10–1.20 mM) was derived from a linear regression of the time-course data of the CTI reaction (0–30 min). (b) Hanes-Woolf double-reciprocal plot of the reaction. All reactions were conducted at the optimal reaction pH and temperature.

Fig. 7. Predicted three-dimensional structure model of the *cis/trans* fatty acid isomerase (CTI) recombinant protein and its proposed catalytic mechanism. Two cysteine residues of the heme-binding motif (CVACH, 46–50 amino acid residues) in the putative cytochrome *c* domain (33–190 amino acid residues, brick red) can be covalently linked to heme by two thioether bonds after cytochrome *c* maturation. (For interpretation of the color, the reader should be referred to the web version of this paper.)

(a)

Forward primer

5' **CAGCCGGCGATGGCCATG** ^{Nco1} CAGGCCCCCAGTCGAG 3'
Overhang sequence Insert sequence

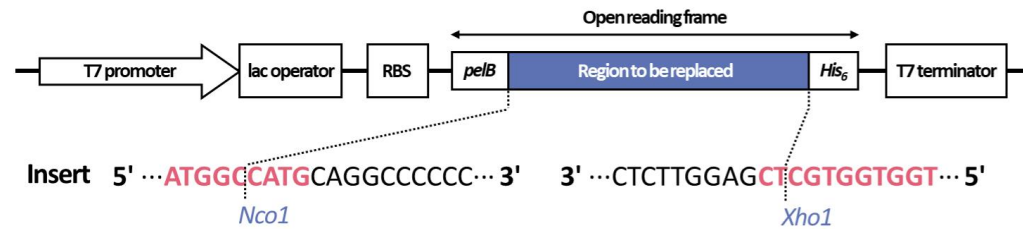
- GC ratio : 74%
- T_m : 58.0°C (with gDNA)
- Base count : 35

Reverse primer

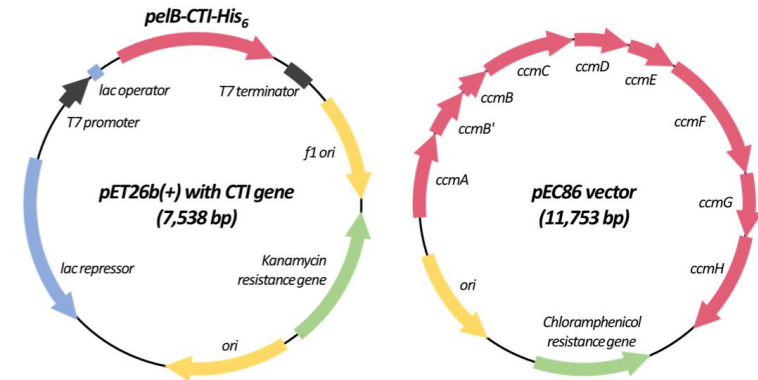
5' **GTGGTGGTGGTGGTGCTC** ^{Xho1} GAGGTTCTCGTAGCGGTTTC 3'
Overhang sequence Insert sequence

- GC ratio : 62%
- T_m : 56.0°C (with gDNA)
- Base count : 37

(b)



(c)



(d)

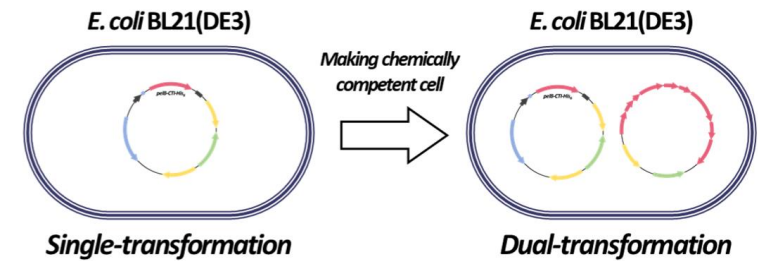


Fig. 1.

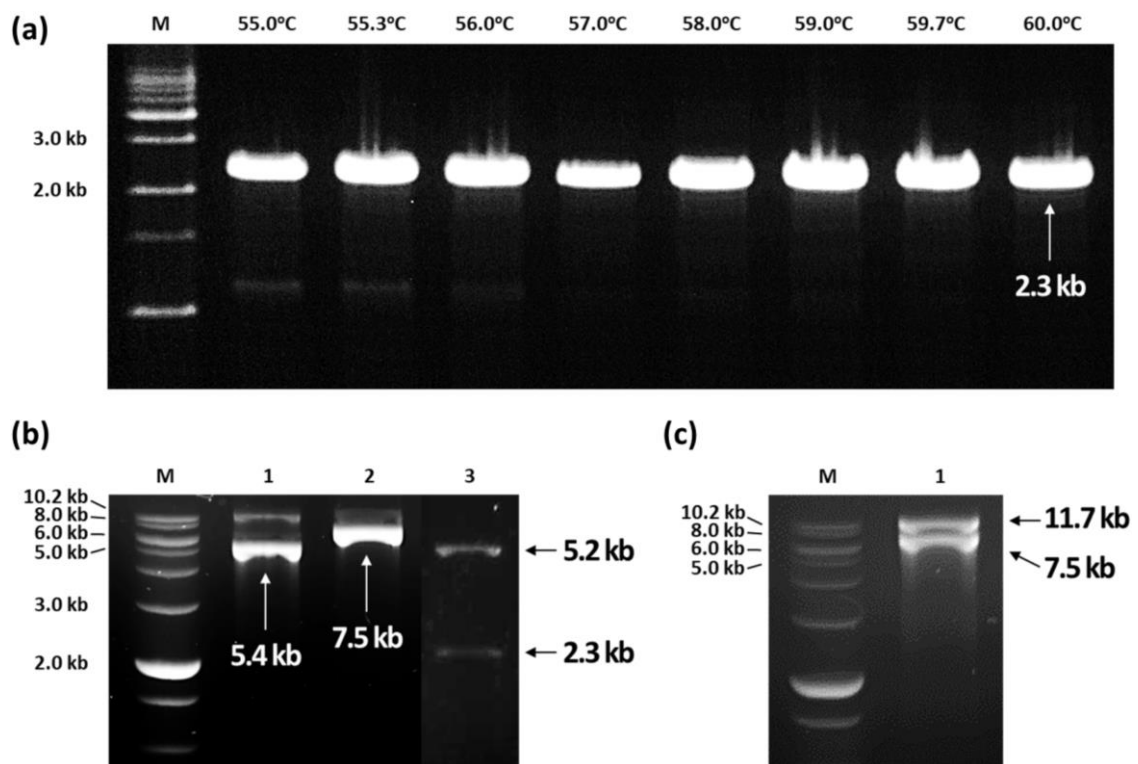


Fig. 2.

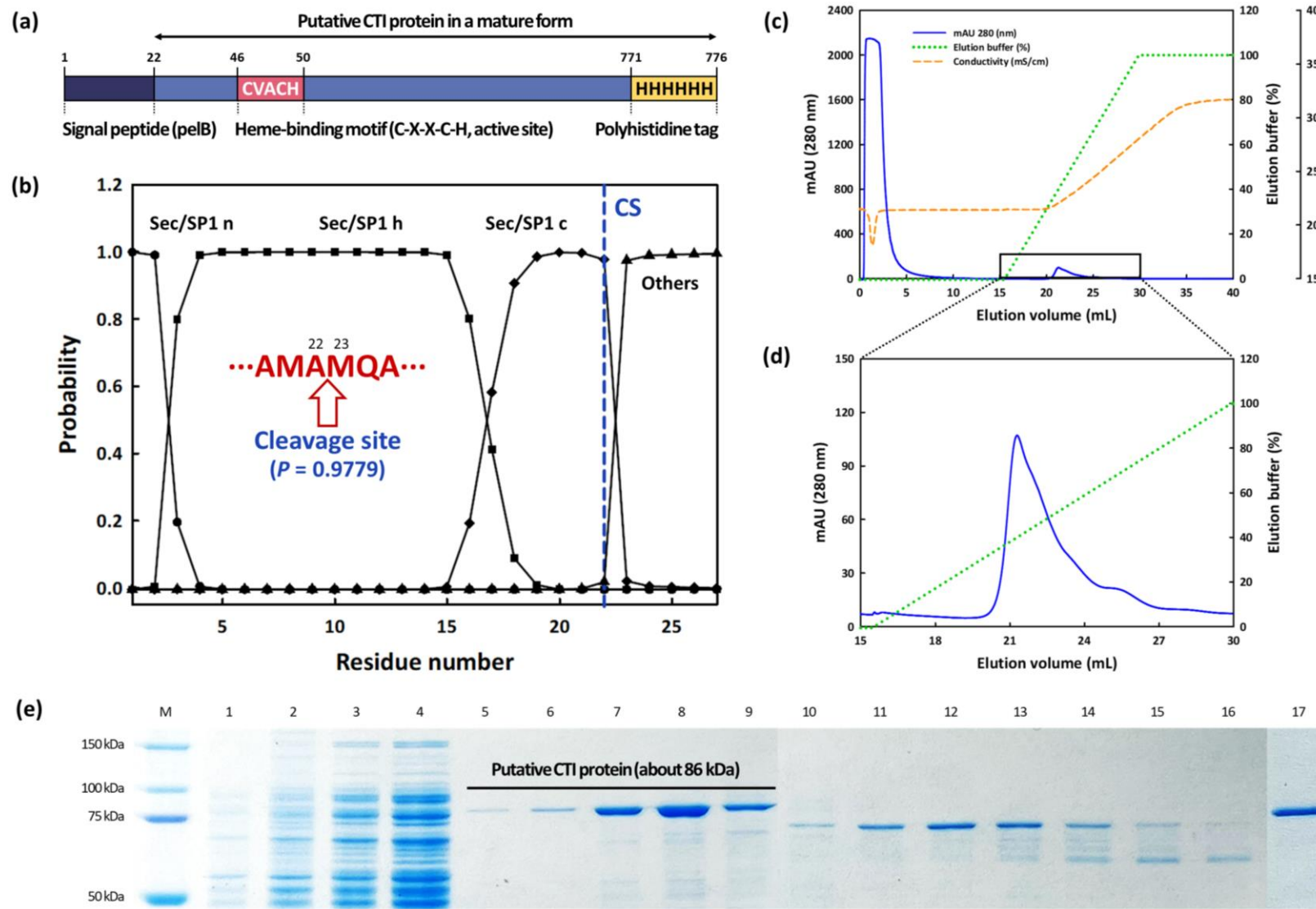


Fig. 3.

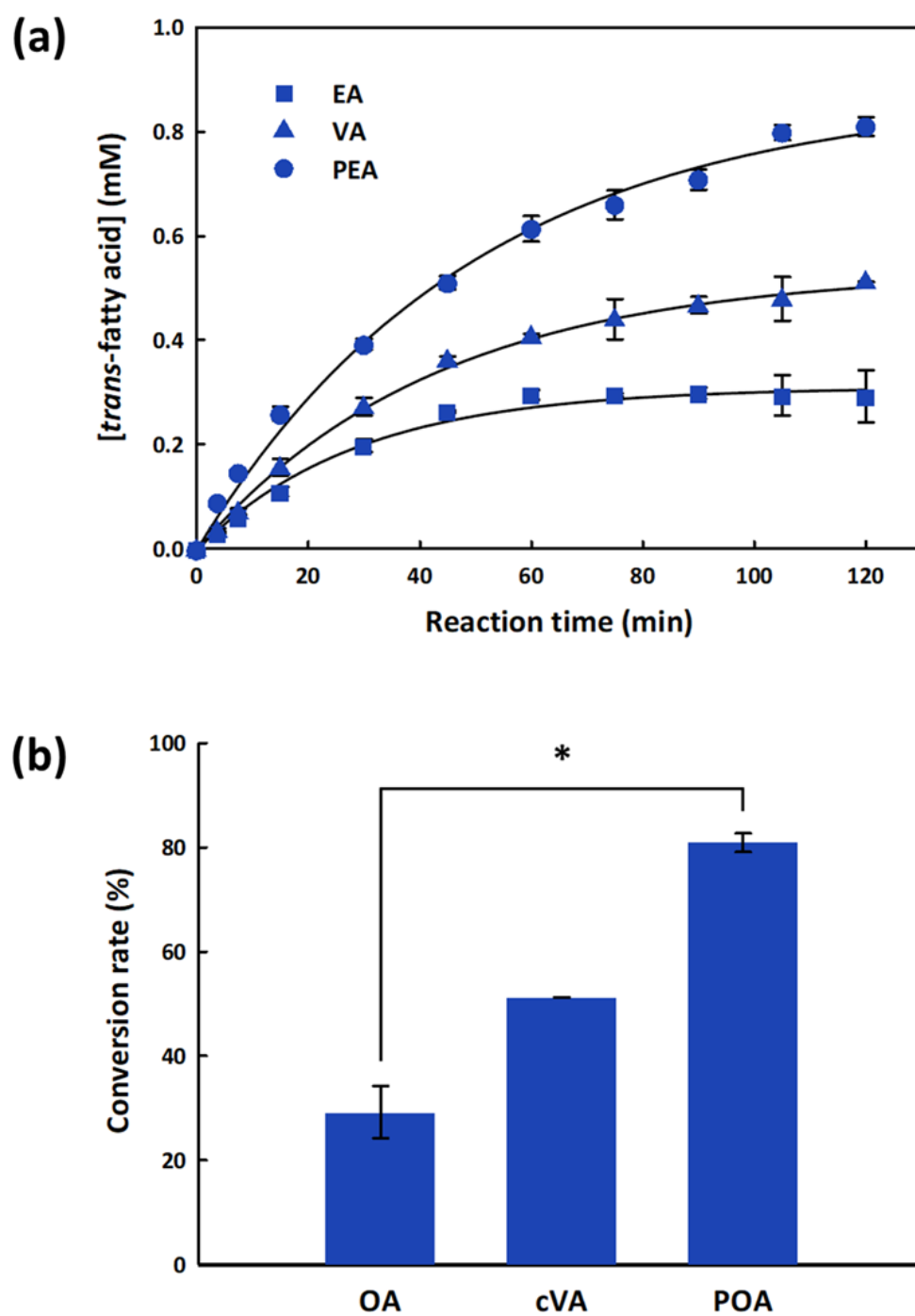


Fig. 4.

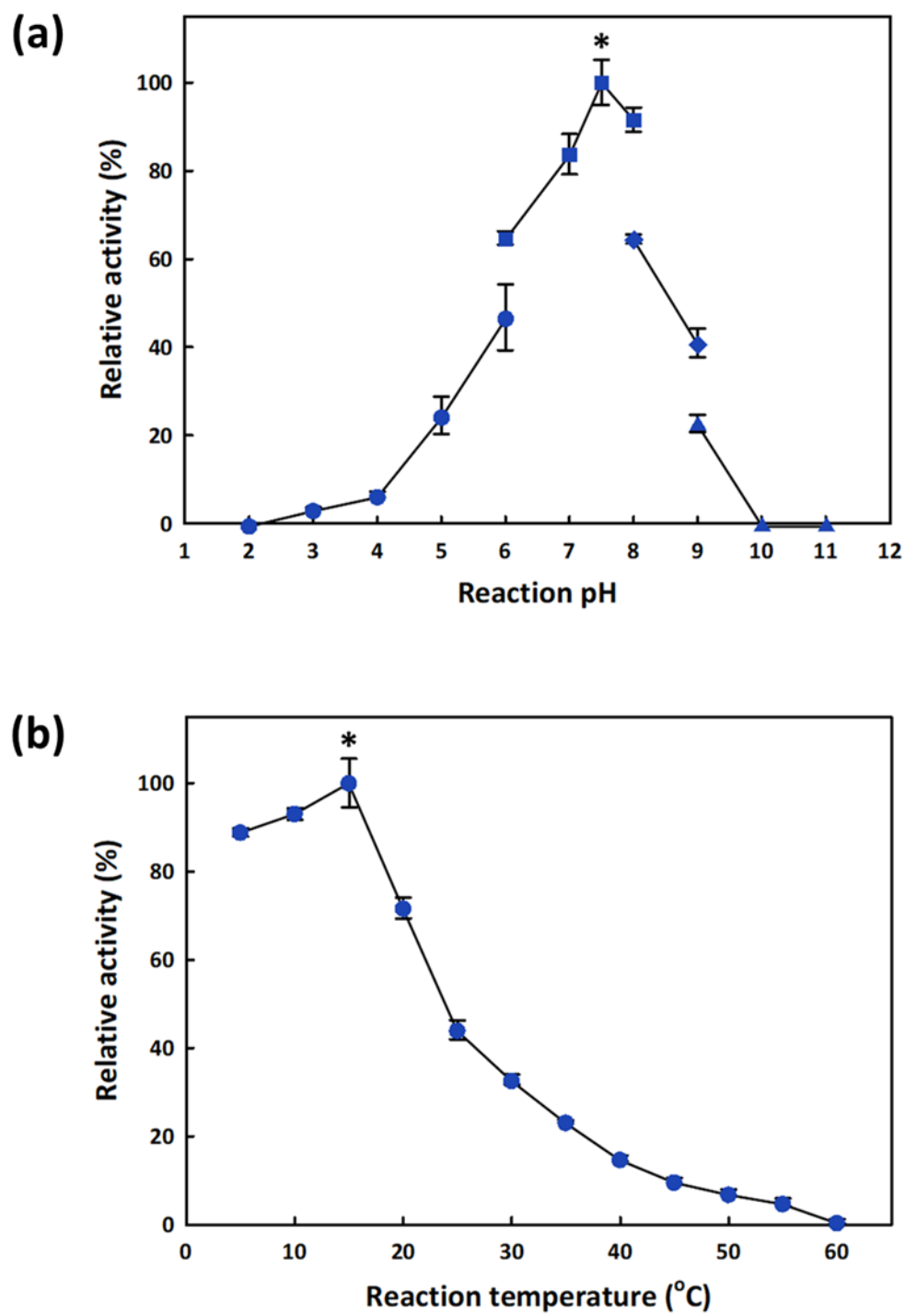


Fig. 5

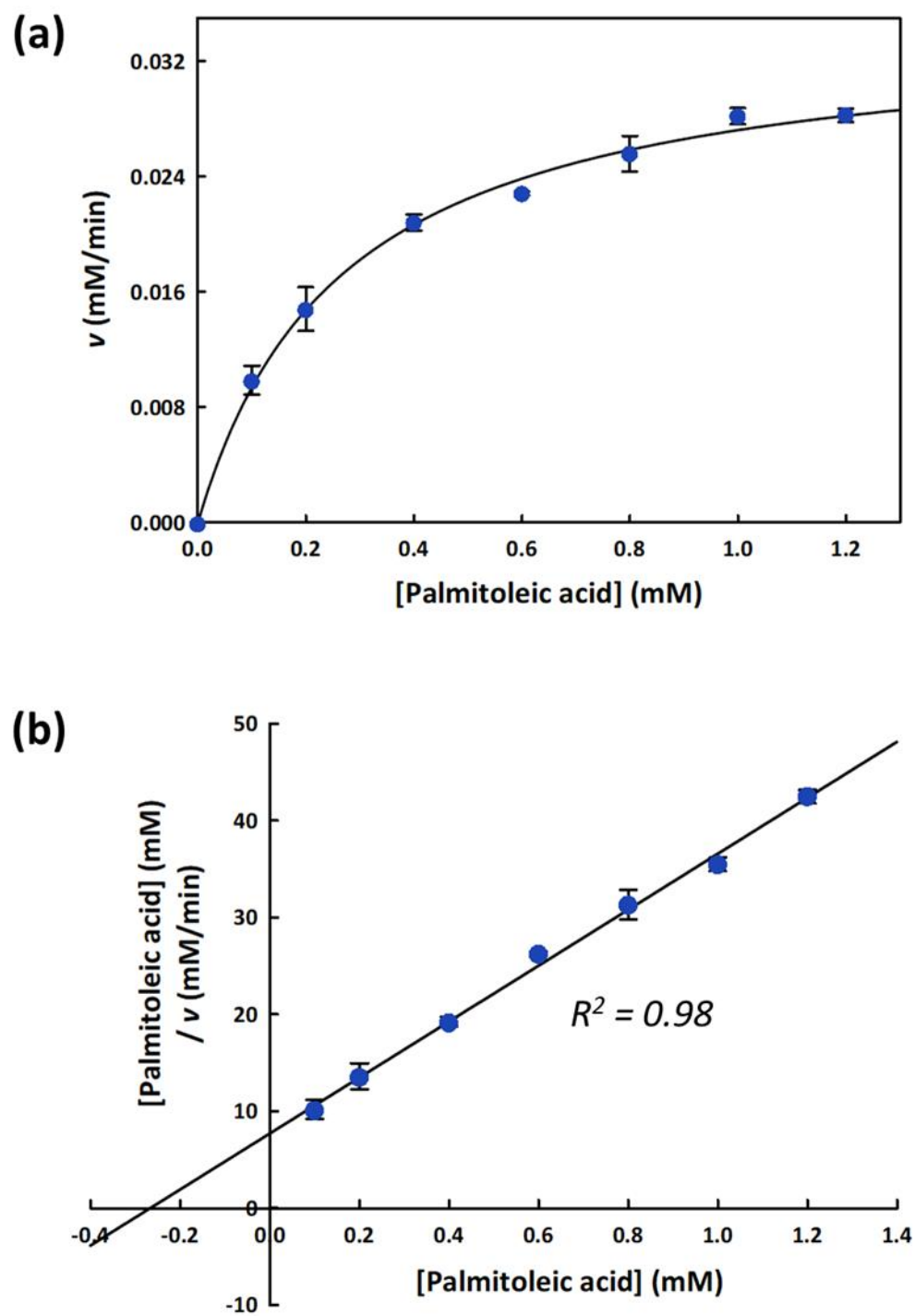


Fig. 6

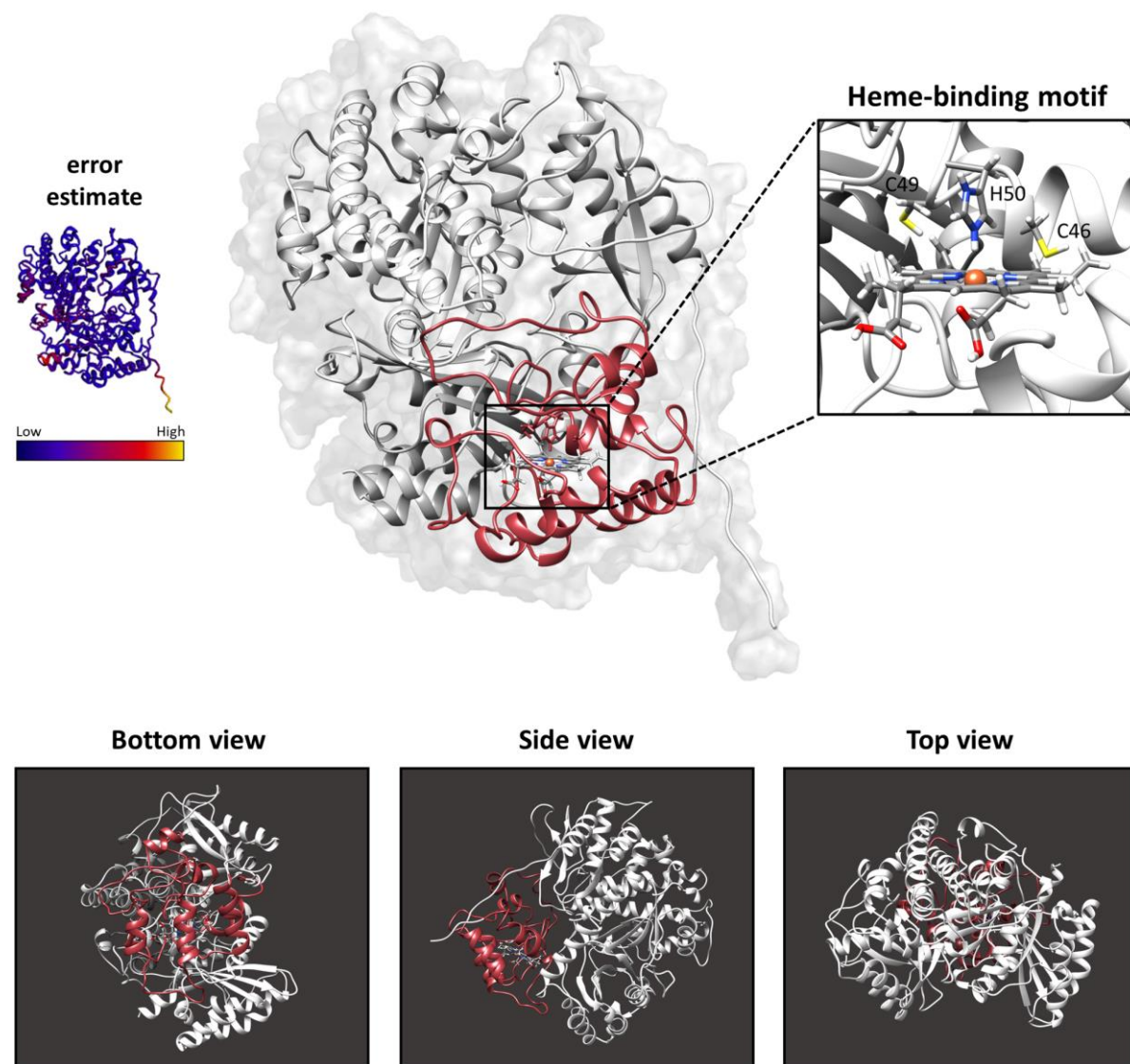


Fig. 7.

Highlights

- *Cis/trans* fatty acid isomerase (CTI) is a bacterial cytochrome *c*-type hemoprotein.
- The recombinant CTI was expressed in the pET26b/pEC86 co-expression system.
- The recombinant CTI showed substrate promiscuity towards unsaturated fatty acids.
- The recombinant CTI showed strong substrate selectivity against palmitoleic acid.
- Deep learning-based structural prediction demonstrated the mode of action of CTI.

Declaration of Interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRedit Authorship Contribution Statement

Jun-Young Park: Conceptualization, Investigation, Data curation, Writing - Original draft, Writing - Review & Editing, Visualization. **Yun-Seo Jung:** Investigation, Validation, Formal analysis, Writing - Review & Editing. **Dimitris Charalampopoulos:** Writing - Review & Editing. **Kyung-Min Park:** Writing - Review & Editing, Project administration, Funding acquisition. **Pahn-Shick Chang:** Conceptualization, Writing - Review & Editing, Supervision, Project administration.

Controlling *cis/trans* isomerism of monounsaturated fatty acids via a recombinant cytochrome *c*-type *cis/trans* fatty acid isomerase

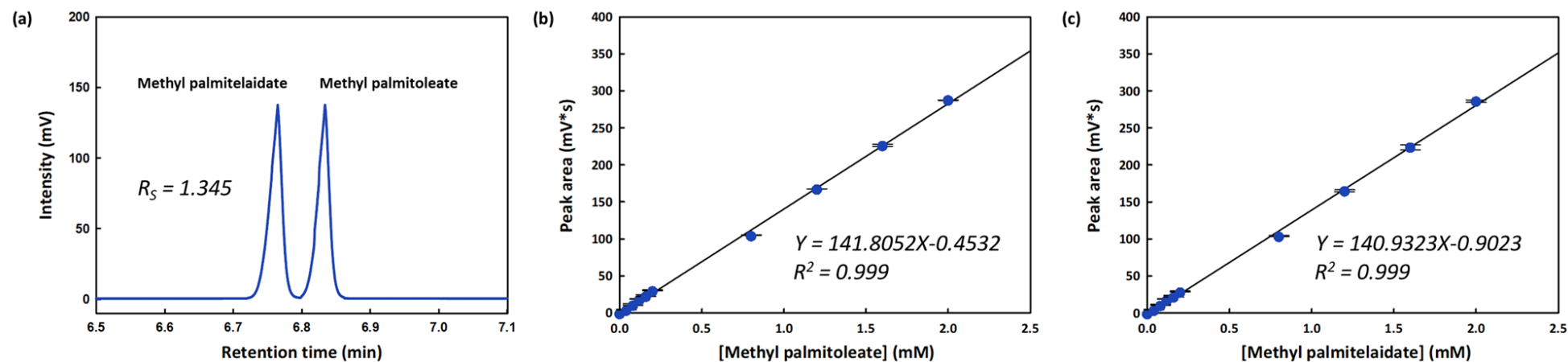
Jun-Young Park¹, Yun-Seo Jung¹, Dimitris Charalampopoulos,

Kyung-Min Park^{*}, Pahn-Shick Chang^{*}

This document file contains Supplementary Data (S1–S8).

Supplementary Data Table S1. The detailed operating condition for the gas chromatography-flame ionization detector (GC-FID) analysis

Parameters	Conditions
GC equipment	YL6500GC system
Column	DB-FastFAME
Length/internal diameter	30 m/0.25 mm
Film thickness	0.25 μ m
Carrier gas	Helium
Mode	1.0 mL/min (constant flow mode)
Inlet	
Temperature	250°C
Split ratio	50:1
FID	
Temperature	250°C
Gas profile	Hydrogen: 40 mL/min
	Zero-air: 300 mL/min
	Make-up gas (nitrogen): 25 mL/min
Injection volume	1 μ L
Oven temperature	80°C (1.0 min)→40°C/min to 200°C (6.0 min)
Total run time	10 min

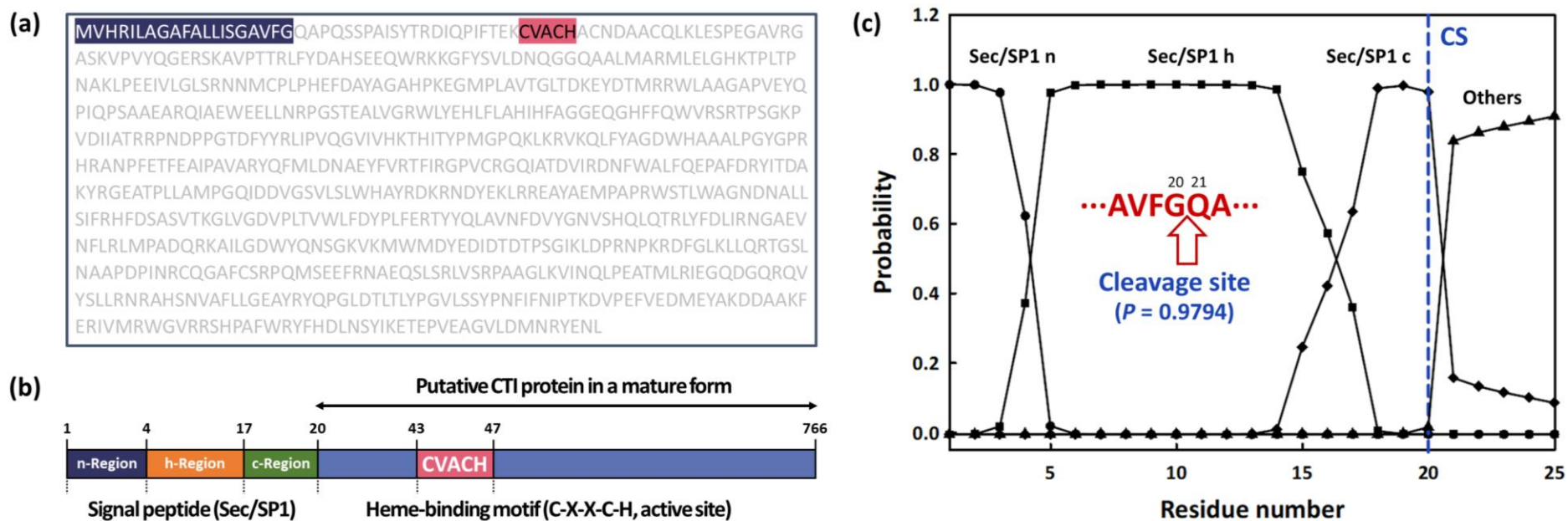


Supplementary Data Fig. S2. Chromatographic resolution of palmitoleic acid and palmitelaidic acid using a GC-FID equipped with a cyanopropyl phase DB-FastFAME capillary column. (a) The GC-FID chromatogram of the mixture of methyl palmitoleate and methyl palmitelaidate dissolved in *n*-hexane at the same molar concentration (1 mM). (b) Calibration curve of methyl palmitoleate. (c) Calibration curve of methyl palmitelaidate.

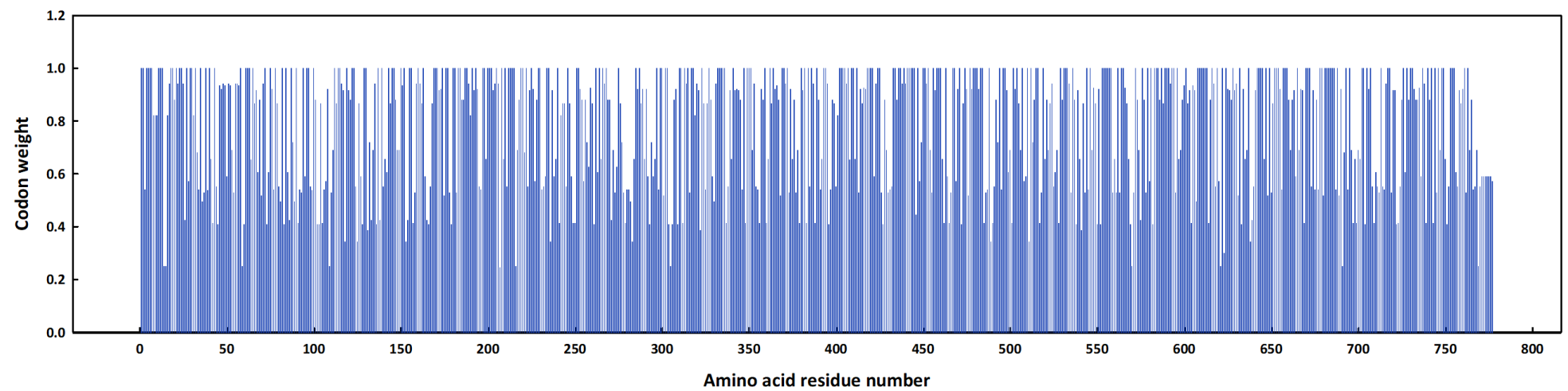
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ACTGA

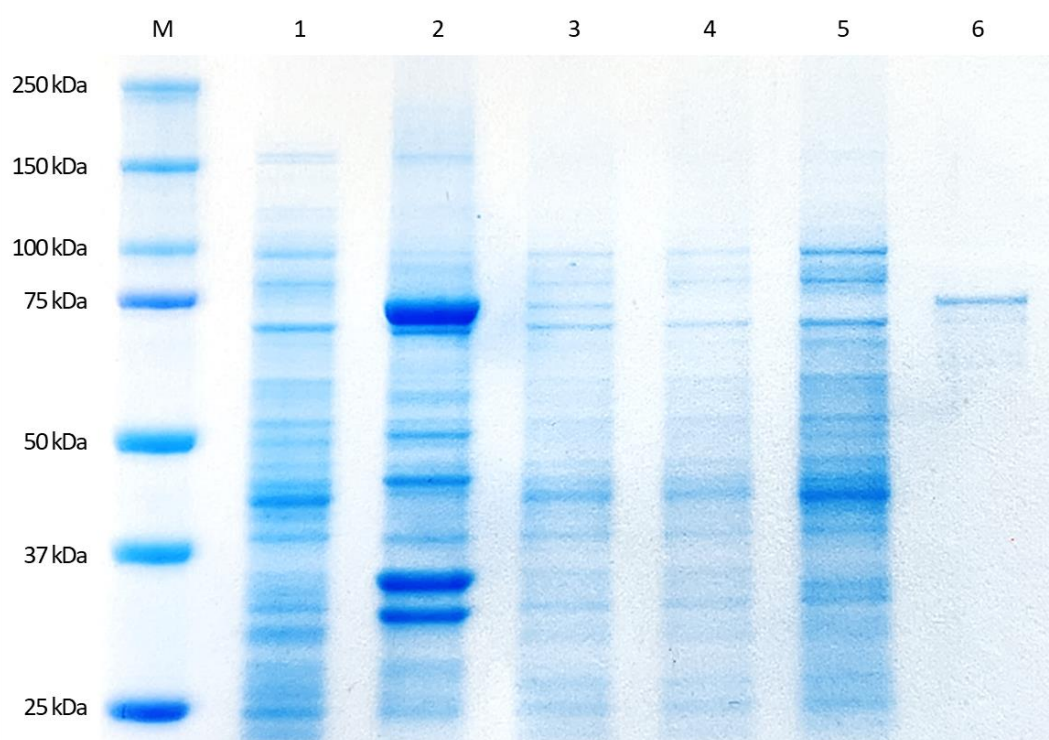
Supplementary Data Fig. S3. Nucleotide sequence of an open reading frame for the *cis/trans* fatty acid isomerase recombinant gene in the constructed pET26b(+) plasmid.



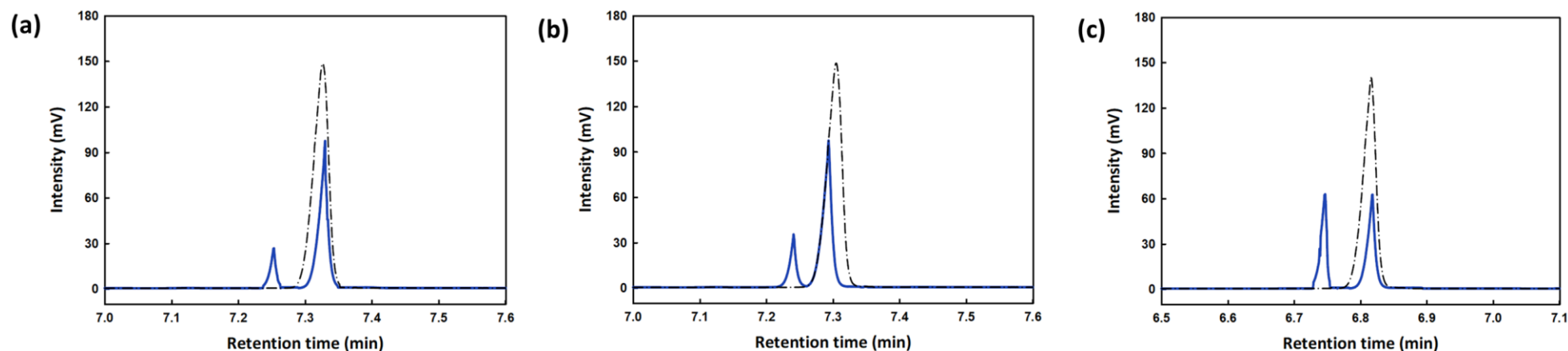
Supplementary Data Fig. S4. Genome-based structural analysis of wildtype *cis/trans* fatty acid isomerase (CTI) from *Pseudomonas putida* KT2440. (a) Full amino acid sequence of CTI from *P. putida* KT2440. (b) Predicted structural features of CTI. This protein is a putative cytochrome *c*-type protein (87 kDa) with an iron-containing heme binding motif (CVACH, red) and a Sec/SP1 signal peptide (1–20 residues). Sec/SP1, the secretory signal peptides transported by the Sec translocon and cleaved by signal peptidase I. (c) Computational prediction of signal peptide and cleavage site (CS, dash dot line) of CTI. The subregion probability of n-region (circle), h-region (square), c-region (diamond), and non-signal peptide region (triangle) was predicted.



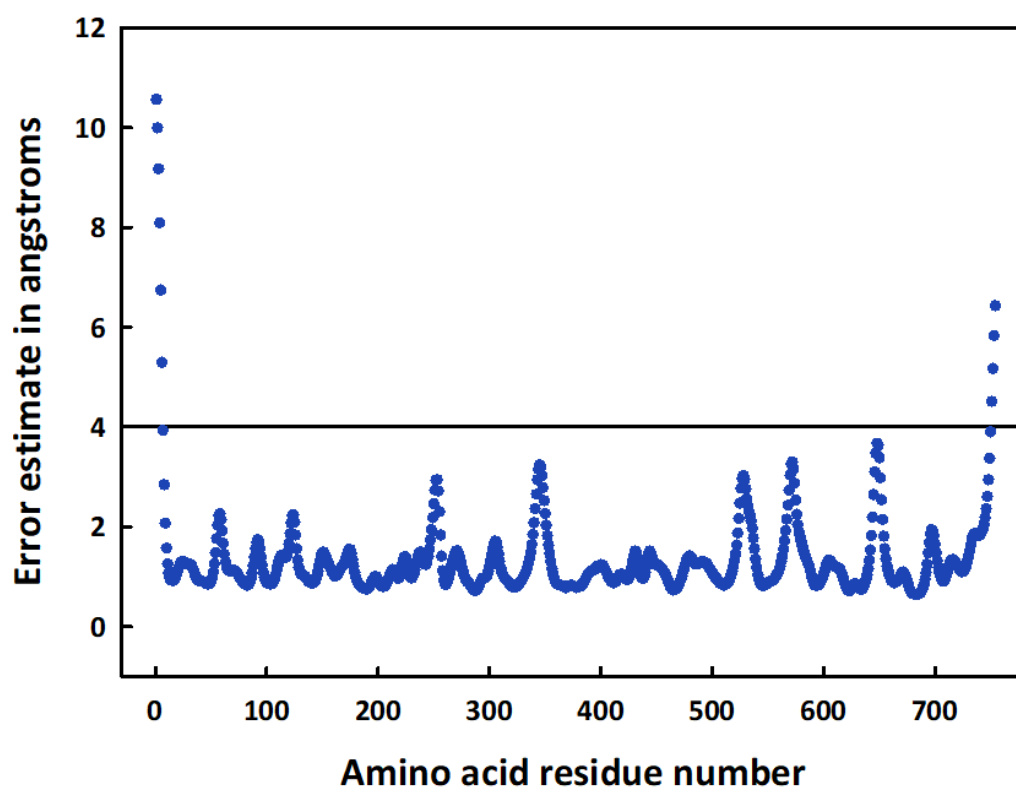
Supplementary Data Fig. S5. Codon weights of each amino acid residue of the *cis/trans* fatty acid isomerase gene cloned in *Escherichia coli* BL21(DE3).



Supplementary Data Fig. S6. Heterologous expression and purification of *cis/trans* fatty acid isomerase from *Pseudomonas putida* KT2440 in the pET26b expression system. The protein bands expected as the recombinant proteins were detected below 75 kDa. M, protein molecular marker; Lane 1, soluble fraction; Lane 2, insoluble fraction; Lane 3, solubilized fraction using a cationic 1%(w/v) CHAPS detergent; Lane 4, unbound fraction (20 mM imidazole); Lane 5, washed fraction (40 mM imidazole); Lane 6, eluted fraction (250 mM imidazole).



Supplementary Data Fig. S7. GC-FID analysis of the reactants from the *cis/trans* isomerization of three monounsaturated fatty acids by *cis/trans* fatty acid isomerase recombinant protein. (a) The GC-FID chromatogram of the methylated fatty acids before (dash dot line) and after (straight line) the 30 min reaction with 1 mM oleic acid ($C_{18:1}$, $cis-\Delta^9$). (b) The GC-FID chromatogram of the methylated fatty acids before (dash dot line) and after (straight line) the 30 min reaction with 1 mM *cis*-vaccenic acid ($C_{18:1}$, $cis-\Delta^{11}$). (c) The GC-FID chromatogram of the methylated fatty acids before (dash dot line) and after (straight line) the 30 min reaction with 1 mM palmitoleic acid ($C_{16:1}$, $cis-\Delta^9$).



Supplementary Data Fig. S8. Angstrom error per amino acid residue in the predicted structure of the *cis/trans* fatty acid isomerase recombinant protein. The predicted structure is considered to be highly confident.

Graphical Abstract

