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Biochemical Characteristization of Propionyl-Coenzyme A Carboxylase Complex of *Streptomyces toxytricini*

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Acyl-CoA carboxylases (ACC) are involved in important primary or secondary metabolic pathways such as fatty acid and/or polyketides synthesis. In the 6.2 kb fragment of *pccB* gene locus of *Streptomyces toxytricini* producing a pancreatic inhibitor lipstatin, 3 distinct subunit genes of presumable propionyl-CoA carboxylase (PCCase) complex, assumed to be one of ACC responsible for the secondary metabolism, were identified along with gene for a biotin protein ligase (Bpl). The subunits of PCCase complex were α subunit (AccA3), β subunit (PccB), and auxiliary ε subunit (PccE). In order to disclose the involvement of the PCCase complex in secondary metabolism, some biochemical characteristics of each subunit as well as their complex were examined. In the test of substrate specificity of the PCCase complex, it was confirmed that this complex showed much higher conversion of propionyl-CoA rather than acetyl-CoA. It implies the enzyme complex could play a main role in the production of methylmalonyl-CoA from propionyl-CoA, which is a precursor of secondary polyketide biosynthesis.

***Keywords*:** propionyl-CoA carboxylase, biotin protein ligase, AccA3, PccB, Bpl, lipstatin, *Streptomyces toxytricini*

Acyl-CoA carboxylase complex catalyzes the first committed step in the biosynthesis of fatty acids and polyketides in animals, plants and bacteria. In other words, this complex is responsible for the activation of various organic acids that can serve as the building blocks for fatty acid or polyketide.

This enzyme complex is composed of three different functional components: the biotin carboxylase (BC) domain, the biotin carboxyl carrier protein (BCCP) domain and the carboxyltransferase (CT) domain (Cronan and Waldrop, 2002; Choi-Rhee and Cronan, 2003). By post-translational event, BCCP domain of apo-enzyme is biotinylated to form holo-enzyme with the aid of biotin protein ligase (Bpl). Then, the first CO_2 fixation on biotin takes place to result in the formation of carboxyl biotin complex by the cooperation of BC and BCCP components. This carboxyl biotin then swings out to the CT component, producing the α-carboxylated acyl-CoA (Fig. 1).

In the genus *Streptomyces* producing a variety of secondary metabolites, two types of acyl-CoA carboxylase were found: acetyl-CoA carboxylase (ACCase) and propionyl-CoA carboxylase (PCCase) (Rodriguez and Gramajo, 1999). Both acyl-CoA carboxylase complexes are composed of α subunit carrying BC and BCCP domain and β subunit having CT domain. By gene disruption study, it was confirmed that ACCase plays a role in primary metabolism for fatty acid synthesis and PCCase in secondary metabolism for polyketide synthesis (Rodrriguez *et al*., 2001; Diacovich *et al*., 2002).

In case of *Streptomyces toxytricini* producing a lipase inhibitor lipstatin, the two acyl-CoA carboxylase gene clusters were also found in the DNA fragments of 11.2 kb and 6.2 kb originated from different genome locus. By sequence analysis of these two fragments, the 9 ORFs were present in 11.2 kb DNA fragment referred as *accD1* gene locus, which encodes the ACCase subunits related to the primary metabolism (Demirev *et al*., 2009).

Another 6.2 kb DNA fragment referred as *pccB* gene locus is comprised of 5 ORFs including the *accA3* encoding α subunits, *pccB* gene for β subunit and *pccE* for ε subunit of PCCase along with *bpl* gene responsible for post-translational biotinylation, (Demirev *et al*., 2010). By gene disruption study of *pccB* in *S. toxytricini*, this gene locus was assumed to be involved in the secondary metabolism for lipstatin biosynthesis. In this study, the genes in *pccB* locus were subcloned and expressed, and the purified recombinant proteins were biochemically characterized in order to disclose the involvement in the production of secondary metabolites.

Materials and Methods

Cultivation of microorganisms

Escherichia coli JM109 and BL21(DE3) served as hosts for routine cloning of DNA and expression of recombinant proteins. The host cells were grown on selective Luria-Bertani (LB) media at 37°C. According to the resistant marker present in the plasmid, the final concentration of 100 μg/ml of ampicillin or 50 μg/ml of kanamycin was supplied.

General DNA manipulation

The general procedures for gene amplification by polymerase chain reaction (PCR), plasmid preparation and transformation, gene cloning, and plasmid confirmation was performed following the published

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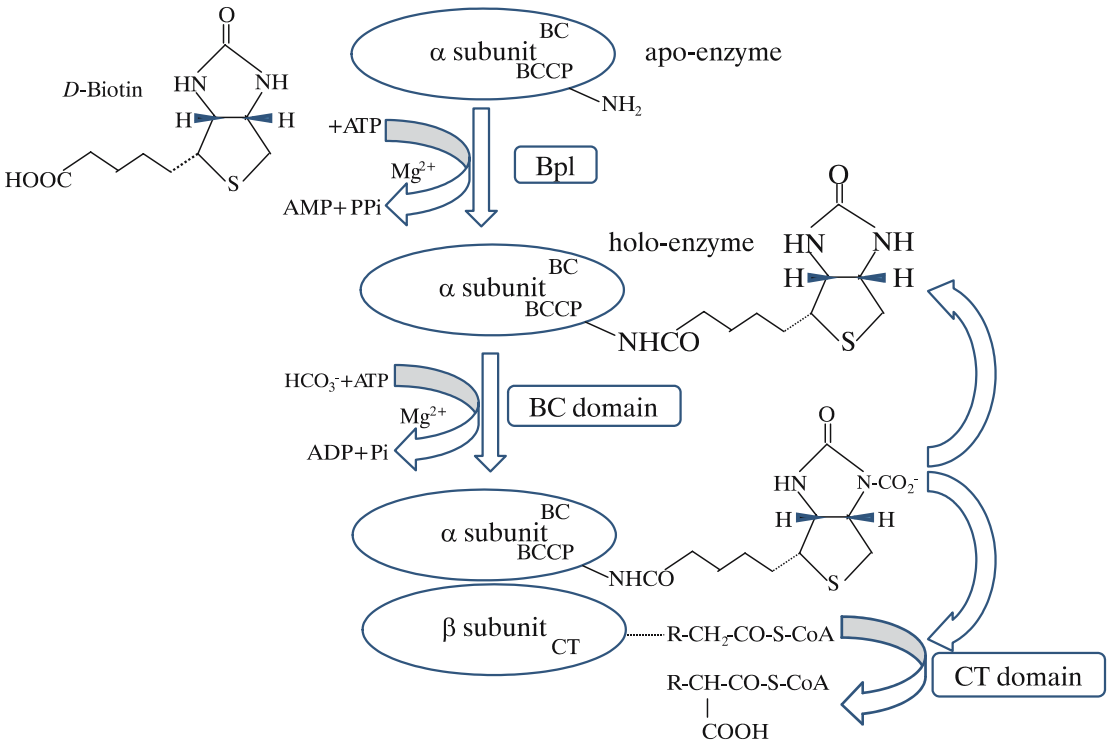

Fig. 1. The graphic scheme for α-carboxylation reaction catalyzed by acyl-CoA carboxylase complex.

protocols (Sambrook and Russell, 2001; Kieser *et al.*, 2000). The primer sets used in this study were AccA3-F (5′-GACGCGACATATGCGCA AGGTGCTCATCGCCA-3′) and AccA3-R (5′-GAGGACTCGAGG TCCTTGATCTCGCAGATCGTC-3′) for *accA3* gene amplification; PccB-F (5′-GACCTCCATATGTCACAACCGTCC GAGCCGATCG ACA-3′) and PccB-R (5′-GTCTGACTCGAGTGAGGGGGATGTT GCCGTGCTTCTTCG-3′) for *pccB* gene amplification; and Bpl-F (5′-AGCCATATGACGCCATCCGATGCCCCAGGCGGGGCT-3′) and Bpl-R (5′-GTGCTCGAGCCCGGCAGGCCTCAGGTGCACC ACGT-3′) for *Bpl* gene amplification. The amplified genes were cloned at *Nde*I and *Xho*I site of T&A plasmid and confirmed by restriction analysis and nucleotide sequencing. The *accA3*, *pccB*, and *bpl* genes were then subcloned between *Nde*I and *Xho*I sites of pET28a(+) expression vector. *E. coli* BL21(DE3) was employed as a host cell of pET28a(+) plasmid for gene expression.

Protein expression, purification, and analysis

The recombinant proteins were expressed by the addition of 0.5 mM isopropyl-β-thiogalactoside (IPTG) at 28°C when OD_{600} of host cell reached around 0.8, and further cultivated for 4-6 h. After sonication, the His_6-tagged AccA3, PccB, and Bpl proteins were purified from the soluble fractions by column chromatography using NTA chelating agarose CL-6B resin (Peptron Inc., Korea) (Demirev *et al.*, 2009). The purified proteins were confirmed by 12% sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (SDS-PAGE), and assayed according to Bradford method (1976).

Detection and exclusion of *in vivo* biotinylated proteins

Streptavidin agarose bead slurry (50% slurry suspension) (Novagen, USA) were used for detection and exlusion of the *in vivo* biotinylated holo-AccA3 according to the previous report (Rybak *et al.*, 2004; Demirev *et al.*, 2009). This procedure is required for the separation of the non-biotinylated apo-AccA3 (α subunit).

In vitro biotinylation assay

To confirm the enzyme activity, the separated apo-AccA3 was *in vitro* biotinylated by Bpl as described in the previous report (Demirev *et al.*, 2009). The standard reaction mixture (100 μl) contained 20 mM glycine-NaOH (pH 11.0), 5.5 mM $MgCl_2$, 10 mM KCl, 3 mM ATP, 0.03 mM or 0.06 mM *D*-biotin, 2.5 μM apo-AccA3, and 60 nM Bpl. The biotinylation reaction proceeded at 30°C until 90 min. The biotinylated holo-AccA3 was detected on 12% SDS-PAGE by horseradish peroxidase (HRP)-conjugated streptavidin (BD Pharminogen, USA) and visualized with ECL reagent (Pierce, USA) on X-ray film.

Propionyl-CoA carboxylase assay

The acyl-CoA carboxylase activity was measured following the incorporation of biocarbonate (HCO_3^-) on acyl-CoA (Kimura *et al.*, 1998; Rodriguez and Gramajo, 1999; Rodriguez *et al.*, 2001; Diacovich *et al.*, 2002). The reaction mixture (100 μl) was composed of 100 mM phosphate buffer (pH 8.0), 100 mM sodium carbonate, 3 mM ATP, 5 mM $MgCl_2$, 300 μg bovine serum albumin, and enzyme complex (1.25 μM holo-AccA3 and 1.25 μM PccB). The reaction was carried out at 30°C for 1 h, and stopped by immersing in boiling water. For the analysis of reaction products, the resultant solution was subjected to high performance liquid chromatography (HPLC) ($SCL\text{-}10A_{VP}$ system, Shimadzu Co., Japan) using YMC-Pack Pro C_{18} reverse-phase column (S-5 μm, 250 mm×4.6 mm I.D., 12 nm) (YMC Co., Ltd., Japan). The mobile phase of 0.1 M acetate buffer (pH 5.0)-methanol [70:30 (v/v)] was pumped at 0.8 ml/min (Kimura *et al.*, 1998). The injection volume was 20 μl and the wavelength for ultraviolet detection was 254 nm.

Results and Discussion

Gene amplification and cloning of acyl-CoA carboxylase components

The gene organization of 6.2 kb DNA fragment in pSTL-24 cosmid screened from genome of *S. toxytricini* NRRL 15,443 revealed the presence of 5 ORFs; *accA3* encoding α subunit (BC and BCCP), *pccB* encoding *β* subunit (CT), *pccE* encoding *ε* subunit, *bpl* encoding protein for post-translational biotinylation, and one putative gene (Demirev *et al.*, 2010).

In order to confirm that those genes are functionally related and form a specific acyl-CoA carboxylase complex involved in the secondary metabolism, the *accA3*, *pccB*, and *bpl* genes were amplified and subcloned. The correct size of 1.7 kb fragment for *accA3,* 1.6 kb for *pccB*, and 0.9 kb for *bpl* were amplified, and subcloned into pET-28a(+) expression vector of *E. coli*.

Expression and purification of acyl-CoA carboxylase components

The overexpression of recombinant proteins in *E. coli* BL21 (DE3) host cell was conducted by gene induction with 0.5 mM IPTG for 4-6 h at 28°C in order to obtain a soluble protein. Since all the recombinant proteins expressed were found to be present in a soluble fraction, the His_6-tagged proteins were purified by Ni^{2+}-NTA affinity column chromatography. The finally purified PccB and Bpl proteins showed in a homogenity on SDS-PAGE (Fig. 2).

Further, it was observed that a half of the recombinant AccA3 was expressed as a holo-enzyme already biotinylated in host cells (bound on streptavidine beads) when *D*-biotin was supplemented in culture medium (Fig. 3). It might be due to the *in vivo* biotinylation of apo-AccA3 by the host *E. coli* BirA (a biotin-induced repressor protein A) (Cronan, 1990). However, in the absence of *D*-biotin, the recombinant AccA3 was almost produced as non-biotinylated apo-enzyme. It is comparable to the previous report that the recombinant AccA1 was completely biotinylated in the presence of *D*-biotin

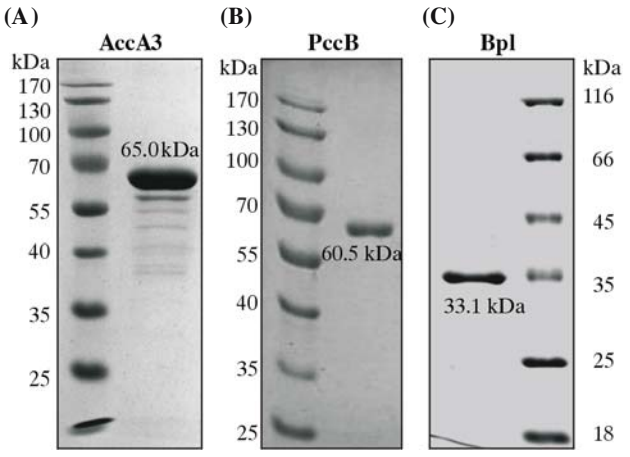

Fig. 2. The purified recombinant proteins of propionyl-CoA carboxylase components. All the recombinant proteins were expressed in *E. coli* BL21(DE) cells, and purified through Ni^{2+}-NTA affinity column chromatography.

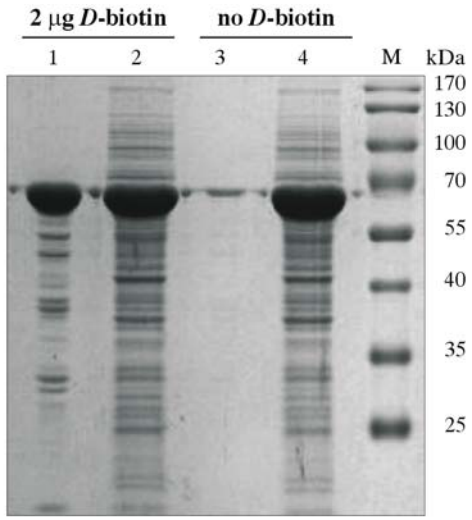

Fig. 3. *In vivo* biotinylation pattern of AccA3 (α subunit) by host BirA protein of *E. coli*. The soluble proteins in *E. coli* lysate carrying *accA3* gene were treated with streptavidin agarose beads in order to confirm the *in vivo* biotinylated proteins by host BirA protein. Lanes: 1, holo-AccA3 (bound on the streptavidin beads) produced when supplemented 2 μg of *D*-biotin in culture media; 2, apo-AccA3 (not bound on the streptavidin beads) produced when supplemented 2 μg of *D*-biotin; 3, holo-AccA3 (bound on the streptavidin beads) produced without supplementation of *D*-biotin; 4, holo-AccA3 (not bound on the streptavidin beads) produced without supplementation *D*-biotin.

(Demirev *et al*., 2009). In another word, the host BirA protein biotinylates AccA1 very well, but not AccA3. It might be an indirect evidence for the involvement of AccA1 in primary metabolism which can be easily biotinylated by *E. coli* BirA (Barker and Campbell, 1981). But poor *in vivo* biotinylation

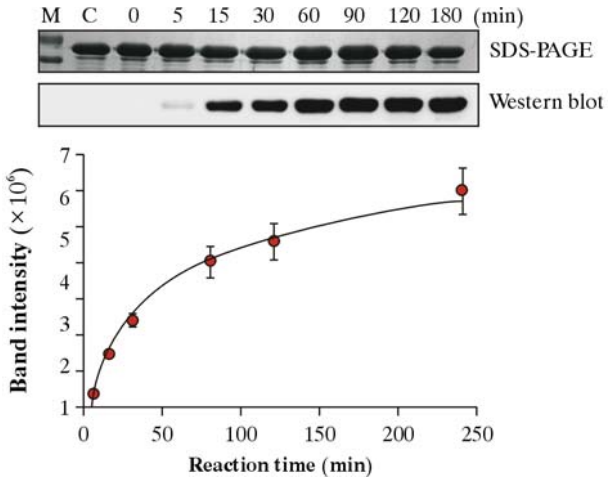

Fig. 4. The time profile of *in vitro* biotinylation reaction of apo-AccA3 by Bpl protein of *S. toxytricini*. *In vitro* biotinylation reaction of apo-AccA3 (2.5 μM) was performed by 0.06 μM Bpl in the presence of 0.03 mM *D*-biotin, 3 mM ATP, 10 mM KCl, and 5.5 mM $MgCl_2$ in glycine buffer (pH 11.0) at 30°C.

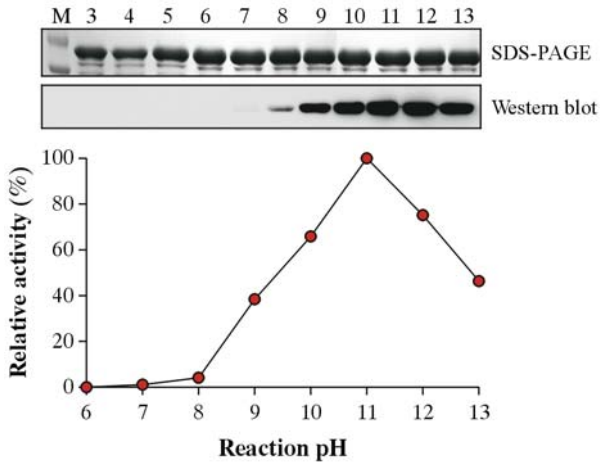

Fig. 5. The pH dependency of *in vitro* biotinylation reaction of apo-AccA3 by Bpl protein of *S. toxytricini*. *In vitro* biotinylation reaction of apo-AccA3 (2.5 μM) was performed by 0.06 μM in the presence of 0.03 mM *D*-biotin, 3 mM ATP, 10 mM KCl, and 5.5 mM $MgCl_2$ in 20 mM different buffers at 30°C for 90 min. The buffers used were phosphate buffer at pH 6 and 7, Tris-HCl buffer at pH 8, 9, and 10, and glycine buffer at pH 11, 12, and 13.

of AccA3 implies its presumable role for secondary metabolism. Thus for the isolation of the recombinant apo-AccA3 protein, the biotinylated proteins were excluded from cell lysate using streptavidin beads.

Though the biotinylated holo-form was removed before purification, the recombinant AccA3 was not purified in a pure form (Fig. 2). It might be attributed to the promiscuous property of α subunit (AccA3) with host proteins including other carboxylase subunits (Choi-Rhee *et al*., 2004).

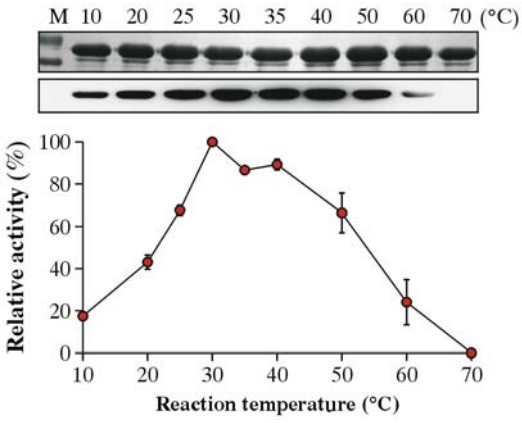

Fig. 6. The temperature dependency of *in vitro* biotinylation reaction of apo-AccA3 by Bpl protein of *S. toxytricini*. *In vitro* biotinylation reaction of apo-AccA3 (2.5 μM) was performed by 0.06 μM Bpl in the presence of 0.03 mM *D*-biotin, 3 mM ATP, 10 mM KCl, and 5.5 mM $MgCl_2$ in 20 mM glycine buffer (pH 11.0) at different temperature for 90 min.

In vitro biotinylation of propionyl-CoA carboxylase

The first step of acyl-CoA carboxylase reaction is the activation of apo-enzyme to holo-enzyme through a post-transcriptional biotinylation by Bpl. Bpl protein transfers a molecule of biotin to the lysine residue in MKM motif at carboxy terminal of BCCP domain in the presence of ATP (Chapman-Smith and Cronan, 1999). To facilitate the biochemical studies of PCCase complex, the *in vitro* biotinylation of the recombinant apo-protein of α subunit (apo-AccA3) was conducted.

The biotinylation reaction of apo-AccA3 proceeded in a time-dependent manner until 1 h (Fig. 4). The optimal reaction conditions were pH 11 (Fig. 5) and 30°C (Fig. 6). Among divalent cations tested, magnesium ion was the best as a co-factor for biotinylation reaction (Table 1).

Substrate specificity of propionyl-CoA carboxylase complex

In the next step, a molecule of CO_2 is transferred to the biotin moiety of α subunit to form carboxyl biotin-enzyme. Subsequently the β subunit (CT) transfers the carboxyl group to the acyl-CoA (Cronan and Waldrop, 2002). Because the acyl-CoA participates only in the second step, the β subunit determines the substrate specificity of this enzyme complex. To confirm the substrate specificity of PccB, the carboxylation assay of PCCase complex composed of holo-AccA3 and PccB was performed using acetyl-CoA or propionyl-CoA as the substrate. The HPLC analysis of reaction product revealed that propionyl-CoA was almost converted to methylmalonyl-CoA within 1 h, but acetyl-CoA was not well to malonyl-CoA (Fig. 7). It is much consistent with *Myxococcus xanthus* PCCase complex which exhibited the higher carboxylation activity for propionyl-CoA, but 10 times lower activity for acetyl-CoA and butyryl-CoA (Kimura *et al*., 1998). Thus it can be concluded that AccA3-PccB complex can be involved in secondary metabolism rather than primary metabolism.

Propionyl-CoA carboxylase complex and lipstatin biosynthesis

In this study, AccA3-PccB complex of *S. toxytricini* was revealed certainly to have a PCCase activity rather than ACCase activity. However, it was not yet confirmed that this enzyme complex can really participate in lipstatin biosynthesis.

Lipstatin, a potent irreversible inhibitor of pancreatic lipase (Weibel *et al*., 1987), is known to be synthesized via Claisen condensation of two fatty acid precursors, octanoic acid and

Table 1. Effect of divalent metal ions on *in vitro* biotinylation of apo-AccA3 by Bpl

Metal ion	Relative activity (%)
Mg^{2+}	100
Mn^{2+}	73.8±16.1
Ca^{2+}	2.4±2.1
Cu^{2+}	11.5±0.6
Zn^{2+}	6.6±3.4
Ni^{2+}	25.0±8.6
Fe^{2+}	8.2±2.1

In vitro biotinylation reaction of apo-AccA3 (2.5 μM) was performed by 0.06 μM Bpl in the presence of 0.03 mM *D*-biotin, 3 mM ATP, 10 mM KCl, and 5.5 mM metal ions in glycine buffer (pH 11.0) at 30°C for 90 min.

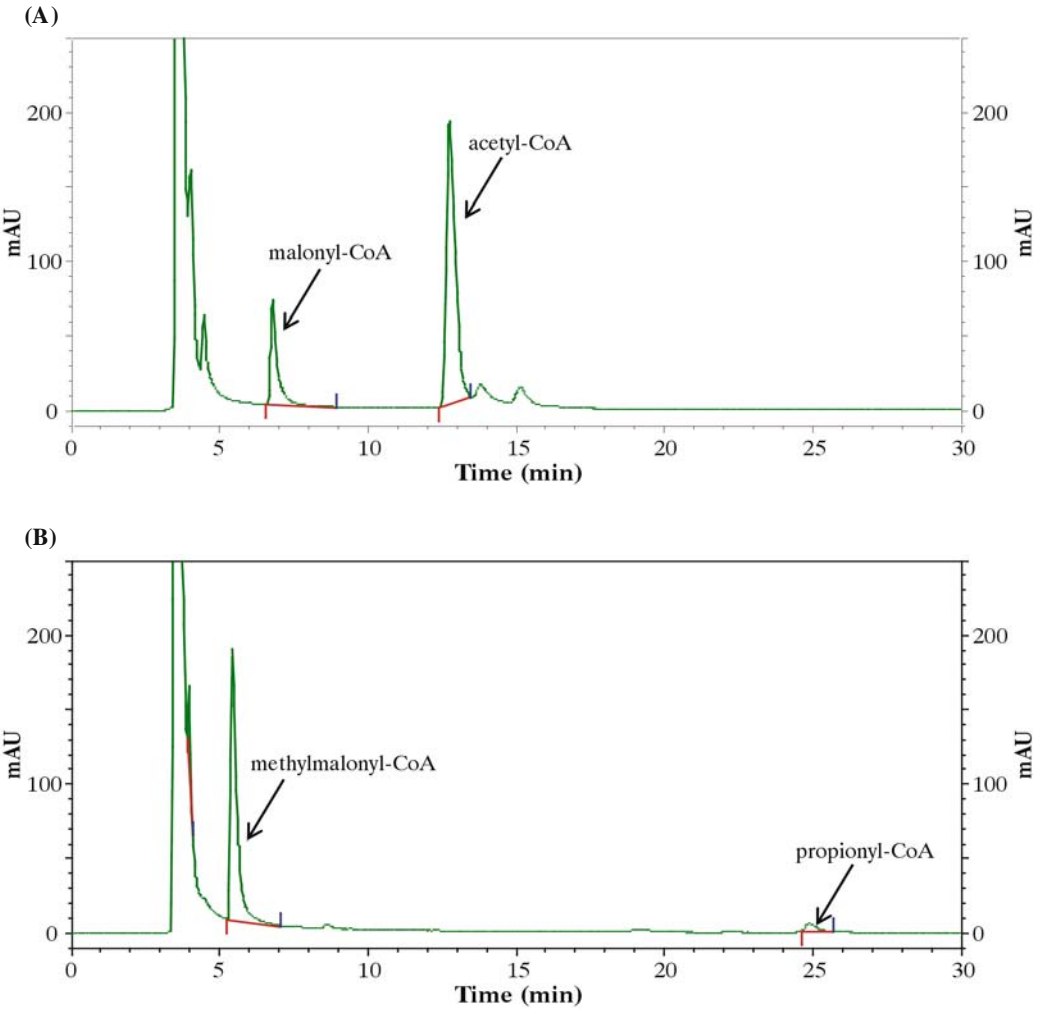

Fig. 7. HPLC analysis of α-carboxylation product catalyzed by propionyl-CoA carboxylase complex of *S. toxytricini*. The α-carboxylation reaction was performed with enzyme complex (1.25 μM holo-AccA3 and PccB each) in 100 mM phosphate buffer (pH 8.0) containing 100 mM sodium carbonate, 3 mM ATP, 5 mM $MgCl_2$, and 300 μg bovine serum albumin at 30°C for 1 h. The reaction products was analyzed by HPLC using C_{18} reverse-phase column. The mobile phase was 0.1M acetate buffer (pH 5.0)-methanol [70:30 (v/v)].

3-hydroxy-5,8-tetradecanoic acid (Goese *et al*., 2001; Schuhr *et al*., 2002; Eisenreich *et al*., 2003). In previous work, the gene disruption experiment of *pccB* suggested the possibility of the involvement of PccB in the activation of octanoic acid to α-carboxy-octanoic acid, which is the second major precursor of lipstatin (Demirev *et al.*, 2010). In spite of this gene disruption result, it is still unclear that PccB can catalyze the α-carboxylation reaction for octanoyl-CoA, because any report on α-carboxylation of long chain acid by bacterial acyl-CoA carboxylase has not been published yet.

The auxiliary ε subunit (PccE) of PCCase in *Streptomyces* has been known to bring a tight interaction of α and β subunits, increasing the specific activity of enzyme complex (Diacovich *et al.*, 2002). However, It is unknown whether the PccE binding to PCCase complex can also contribute on substrate specificity of this complex or not. In order to make clear the possibility of PccB to convert octanoyl-CoA, a further study on α-carboxylation reaction of octanoyl-CoA by PCCase complex in the presence of PccE should be addressed.

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