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Identification and Characterization of Acetyl-CoA Carboxylase Gene Cluster in *Streptomyces toxytricini*

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The gene locus for acetyl-CoA carboxylase (ACC) involved in the primary metabolism was identified from the genomic library of *Streptomyces toxytricini* which produces a lipase inhibitor lipstatin. The 7.4 kb cloned gene was comprised of 5 ORFs including *accD1*, *accA1*, *hmgL*, *fadST1*, and *stsF*. In order to confirm the biochemical characteristics of AccA1, the gene was overexpressed in *Escherichia coli* cells, and the recombinant protein was purified through Ni^{2+} affinity chromatography. Because most of the expressed AccA1 was biotinylated by host *E. coli* BirA in the presence of *D*-biotin, the non-biotinylated apo-AccA1 was purified after gene induction without *D*-biotin, followed by exclusion of holo-AccA1 using streptavidin beads. The separated apo-AccA1 was post-translationally biotinylated by *S. toxytricini* biotin apo-protein ligase (BPL) in a time- and enzyme-dependent manner. This result supports that this gene cluster of *S. toxytricini* encodes the functional ACC enzyme subunits to be biotinylated.

***Keywords*:** acetyl-CoA carboxylase, biotin carboxylase, carboxyltransferase, biotin apo-protein ligase, *Streptomyces toxytricini*

Streptomyces toxytricini produces lipstatin, an irreversible inhibitor of pancreatic lipase (Hochuli *et al*., 1987; Weibel *et al*., 1987). Its carbon skeleton having a unique β-lactone group has been reported to be synthesized via Claisen condensation of two fatty acid precursors (Eisenreich *et al*., 1997, 2003; Goese *et al*., 2000; Schuhr *et al.*, 2002). It resembles the biosynthesis of a mycolic motif formed by activation of one of the acyl precursors by AccD4 (Portevin *et al*., 2005) and Claisen condensation by Pks13 condensase, a non-iterative type I polyketide synthase (Portevin *et al*., 2004).

The attempt to identify *accD4*-like genes in the *S. toxytricini* chromosome led to identification of two gene clusters responsible for acyl-CoA carboxylase (ACCase) complexes (Demirev, 2009). One of the them includes *accA1* and *accD1* which encode the α and β subunits of acetyl-CoA carboxylase (ACC), and the other contains *accA3*, *pccB*, and *pccE* which encode α, β, and ε subunits of the propionyl-CoA carboxylase (PCC) complex and *bpl* gene involved in post-translational modification of α subunits.

Two types of ACCase were already characterized in *S. coelicolor*: ACC involved in primary metabolism for fatty acid synthesis and PCC in secondary metabolism for polyketide synthesis (Rodriguez and Gramajo, 1999; Rodriguez *et al.*, 2001; Gago *et al*., 2006). Thus the *accA1* gene locus is presumed to be responsible for the primary metabolism of *S. toxytricini*, while the *accA3* gene locus responsible for the secondary metabolism (Demirev, 2009).

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This paper reports the organization of the *accA1* gene cluster of *S. toxytricini*. Furthermore, *in vitro* biotinylation of AccA1 by *S. toxytricini* biotin apo-protein ligase (BPL) is also described for better understanding of its biochemical property.

Materials and Methods

Microorganisms, vectors, and cultivation

Escherichia coli DH5α was used for gene manipulation and *E. coli* BL21(DE3) (Novagen, USA) for overexpression of recombinant proteins. These were cultivated on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0). If necessary, 100 μg/ml of ampicillin or 50 μg/ml of kanamycin was added to the medium. *S. toxytricini* NRRL 15443 was maintained in modified MS agar (2% soya bean flour, 2% mannitol, 0.1% glycerol, 0.1% Triton X-100, pH 7.0) or in TSB medium (3% tryptic soy broth) at 29°C for 48 h with aeration. A T&A cloning vector (RBC Bioscience, Taiwan) was employed for cloning the PCR products in *E. coli* DH5α, and a pET-28a(+) vector (Novagen) was utilized for subcloning and expression of *accA1* and *bpl* genes in *E. coli* BL21(DE3). A *Streptomyces* - *E. coli* shuttle cosmid vector pOJ446 (Bierman *et al*., 1992) was used to construct the genomic library. Apramycin (50 μg/ml) was supplemented for selection of cosmid DNAs.

Construction of *S. toxytricini* genomic library

Genomic DNA was isolated from mycelia of *S. toxytricini* using a cetyltrimethylammonium bromide (CTAB) procedure (Kieser *et al*., 2000) and partially digested with *Sau*3AI. The large-size DNA fragments from the agarose gel were ligated

with the pOJ446 cosmid digested with *Hpa*I, dephosphorylated and further cleaved by *Bam*HI. The ligated products were packaged *in vitro* by Gigapack III gold packaging extract (Stratagene, USA), and transfected into *E. coli* XL1-Blue MRF' cells.

DNA manipulations

Plasmid and cosmid DNA isolation, DNA cleavage, ligation, and *E. coli* transformation were performed according to standard methods (Sambrook and Russell, 2001).

PCR amplification

Gene amplification was done using an EF-*Taq* DNA polymerase or *Pfu* DNA polymerase (Solgent, Korea). For the preparation of the carboxyltransferase (CT) probe, PCR was performed at an annealing temperature of 58°C with the combinations of two sets of forward and reverse primers: ACC-F; 5'-SSHBTTCGACSCSGRVTTCTTCG-3' and ACC-R; 5'-RCSAGSGASGASGAGCASGCSGTGTC-3'. For the amplification of the *accA1* gene, forward primer AccA1-F; 5'-CCAGGATCCATGTCCAGCACTGTCCTTGTGGCCAA-3' having *Bam*HI site and reverse primer AccA1-R; 5'-CAAAAGCTTGCGCGCCCGCCTCCTCGTCCTCGTCGG-3' containing *Hin*dIII site were designed. The *bpl* gene was also amplified using forward primer Bpl-F; 5'-CAGCCATATG ACGCCATCCGATGCCCCAGGCGGGGCT-3' having *Nde*I site and reverse primer Bpl-R; 5'-GGTGCTCGAGCCCGG CAGGCCTCAGGTGCACCACGT-3' containing *Xho*I site.

Nucleotide and protein sequence analysis

The selected plasmids were sequenced by Solgent (Korea). The location of ORFs was determined using an ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The homology search of DNA or protein sequences was performed with the BLAST program (http://blast.ncbi.nlm.nih.gov), and the sequence alignment was made using the CLUSTAL W2 program (http://www.ebi.ac.uk/Tools/clustalw2). The nucleotide sequence of the *accA1* locus was deposited under GenBank accession no. FJ618544, and the sequence of the *bpl* gene as a part of the *pccB* gene locus was filed under GenBank accession no. FJ595232.

Gene expression and purification of recombinant proteins

The recombinant *E. coli* BL21(DE3) cells were grown in LB medium supplemented with kanamycin at 28°C until the OD_{600} reached 0.8. Gene expression was induced by 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) and continued for 4~6 h at 27~28°C. In the production of AccA1, the cells were cultured with or without 0.2 ng/ml of *D*-biotin in the LB medium. The harvested cells by centrifugation were resuspended in lysis buffer A (25 mM Tris-HCl; pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, 0.6% N-lauroylsarcosine, and 10% glycerol) and disrupted by lysozyme treatment (50 μg/ml). The soluble proteins were recovered by centrifugation at 16,000 rpm and clarified through membrane filters (0.45 μm). Then the recombinant His_6-tagged proteins were purified by Ni^{2+} affinity column chromatography using NTA chelating agarose CL-6B resin (Peptron Inc., Korea). Selected elution fractions were dialyzed against storage buffer (25 mM Tris; pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol) and then stored at -70°C. The protein purity was assessed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R-250 staining, and the protein concentrations were determined by Bradford assay.

Confirmation and exclusion of *in vivo* biotinylated holo-AccA1

Streptavidin beads (50% slurry, Novagen) were used for the detection of *in vivo* biotinylated holo-AccA1. The soluble proteins (500 μl) in *E. coli* lysate were mixed with an equal volume of 50% streptavidin beads and gently agitated at 4°C for 4 h. After short centrifugation, unbound non-biotinylated apo-AccA1 from the supernatant was collected. The precipitated beads were washed gently with cold water, and the biotinylated proteins on the beads were eluted in 500 μl of 1× sample loading buffer (40 mM Tris; pH 6.8, 0.8% sodium dodecyl sulfate, 10% glycerol, 0.1 M dithiothreitol, 0.01% bromophenol blue). The bound and unbound samples were analyzed on 12% SDS-PAGE, and the proteins were stained with Comassie Brilliant Blue R-250. The supernatant containing apo-AccA1 was aliquoted and kept at -70°C.

In vitro biotinylation of apo-AccA1 and detection of holo-AccA1

The separated apo-AccA1 was biotinylated *in vitro* by *S. toxytricini* BPL using a modified procedure described previously (Chapman-Smith *et al*., 1999). The standard reaction mixtures (100 μl) contained 20 mM Tris-HCl (pH 7.9), 5.5 mM $MgCl_2$, 100 mM KCl, 5 μM dithiothreitol, 3 mM ATP, 1.25 μM apo-AccA1, and 0.03 or 0.06 mM *D*-biotin. The concentration of BPL and the reaction time varied according to the requirement of the analysis. Following incubation at 28°C, the reactions were quenched by adding 900 μl of cold acetone, and the samples were frozen at -70°C for at least 1 h. Then, the precipitated proteins were recovered by centrifugation at 16,000 rpm for 20 min, washed with 1 ml of cold acetone, and completely air-dried for 30 min. After being boiled with 20 μl of 1× sample loading buffer, 15 μl was run on 12% SDS-PAGE to monitor for the amount of the proteins using Coomassie Brilliant Blue R-250, and the remaining 5 μl was loaded on another SDS-PAGE and transferred to a Protran nitrocellulose membrane (Schleicher & Schuell, Germany) using a Mini-transblot II cell (Bio-Rad, USA). The protein blots were soaked in 1× blocking solution containing 5% skim milk in TBST buffer (0.15 M NaCl, 0.1% Tween-20 in 20 mM Tris-HCl buffer, pH 7.6) for 1 h. The membrane was then immersed in 1× blocking solution containing horse radish peroxide (HRP)-conjugated streptavidin (1/5,000 diluted) (BD Pharminogen, USA) for 1 h. After washing 3 times with TBST buffer, the biotinylated products were detected by an enhanced chemiluminescence (ECL) kit (Pierce, USA), followed by X-ray film exposure for 1~5 min. The amount of biotinylated products was quantified using molecular imaging software version 4.0 (Eastman Kodak, Germany).

Results and Discussion

Cloning and sequencing of *accA1* gene cluster

Based on the structural similarity between lipstatin and mycolic motif, the identification of *pks13* gene (type I *pks*-like gene) in *S. toxytricini* was attempted, but any expected result was not obtained (data not shown). Only a type II *pks* gene was previously identified in this strain (Yoo *et al.*, 2006).

In order to identify *accD4*-like gene in the *S. toxytricini* chromosome, the primers were designed based on the sequences of two conserved regions in the β subunit of acyl-CoA carboxylase (CT) and employed for the amplification of probe from chromosomal DNA. The amplified 530 bp CT probe was radiolabeled for the Southern blot analysis of *S. toxytricini* chromosomal DNA. The band of 7.4 kb from *Bam*HI digestion gave a strong signal (Fig. 1).

After partial digestion with *Sau*3AI, a *S. toxytricini* genomic library was constructed in a pOJ446 cosmid and screened by hybridization with the radiolabeled probe. Three cosmid clones having a full-sized 7.4 kb DNA fragment in Southern blotting after *Bam*HI digestion were selected. The 7.4 kb *Bam*HI-digested fragment of the pSTL1 cosmid, one of the positive cosmids, was further digested with *Pst*I to give 3 smaller fragments of 3.6 kb, 2.6 kb, and 1.2 kb, which were subcloned into pGEM-3Zf(+). The nucleotide sequences of these DNA inserts were determined and assembled manually in a single 7.4 kb contig sequence.

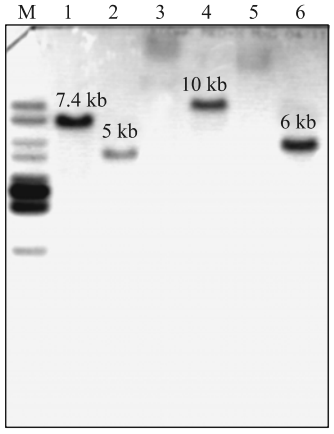

Fig. 1. Southern blotting of *S. toxytricini* chromosomal DNA with CT probe (*accD* probe) on a 0.8% agarose gel. Lanes: 1, *Bam*HI-digested *S. toxytricini* chromosomal DNA; 2, *Pst*I-digested DNA; 3, *Hin*dIII-digested DNA; 4, *Nco*I-digested DNA; 5, *Bgl*II-digested DNA; 6, *Pvu*I-digested DNA; M, 1 kb DNA ladder.

Gene organization of *accA1* gene cluster

The gene organization and orientation in the 7.4 kb DNA were analyzed, and the putative function of the gene products was deduced using the BLAST search program (Fig. 2 and Table 1). Five ORFs were found including *accD1*, *accA1*, *hmgL*, *fadST1*, and *stsF*, and all genes except *stsF* were in the same direction under one promoter. The gene organization of the *accA1* locus in *S. toxytricini* is quite similar with that of *S. avermetilis* (NC003155) and *S. coelicolor* (NC003888).

The *accD1* gene encodes a 58.1 kDa protein (538 amino acids, AccD1) named as the β subunit of ACC (CT). AccD1 showed a high degree of similarity with other β subunits of ACCases from streptomycetes including *S. avermitilis*, *S. coelicolor*, *S. clavuligerus*, *S. griseus*, *S. pristinaespiralis*, *S. sviceus*, and *S.* sp. Mg1. The analysis of AccD1 elucidated the presence of $GG^{149\text{-}150}$ residues binding with the carbonyl group

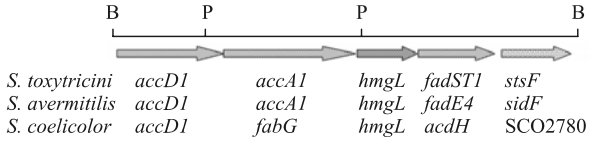

Fig. 2. Gene organization of the *accA1* locus of *S. toxytricini* chromosomal DNA. The gene signatures are *accD1* for CT, *accA1* for BC, *hmgL* for hydroxymethylglutaryl-CoA lyase, *fadST1* for acyl-CoA dehydrogenase, and *stsF* for ferrichrome ABC transporter. The restriction sites are denoted as B for *Bam*HI and P for *Pst*I. The gene organization was compared with a similar locus of *S. avermetilis* (NC003155) and *S. coelicolor* (NC003888).

Table 1. The deduced ORFs in *accA1* gene locus by BLAST search

ORF	Nucleotides position	Amino acids	Gene designation	The closest gene from the BLAST search	Homology (%)
Orf1	26-1642	538	*accD1*	acetyl/propionyl CoA carboxylase, β subunit [*Streptomyces* sp. Mg1]	95
				acetyl/propionyl CoA carboxylase, β subunit [*S. pristinaespiralis* ATCC 25486]	93
Orf2	1673-3694	673	*accA1*	acetyl/propionyl CoA carboxylase, α subunit [*Streptomyces* sp. Mg1]	85
				acetyl/propionyl CoA carboxylase, α subunit [*S. sviceus* ATCC 29083]	78
Orf3	3706-4632	308	*hmgL*	hydroxymethylglutaryl-CoA lyase [*Streptomyces* sp. Mg1]	88
				putative hydroxymethylglutaryl-CoA lyase [*S. griseus* subsp. *griseus* NBRC 13350]	85
Orf4	4640-5800	386	*fadST1*	acyl-CoA dehydrogenase [*Streptomyces* sp. Mg1]	94
				acyl-CoA dehydrogenase [*S. pristinaespiralis* ATCC 25486]	92
Orf5	6119-7180	353	*stsF*	ferrichrome ABC transporter substrate-binding protein [*Streptomyces* sp. Mg1]	88
				ferrichrome ABC transporter [*S. pristinaespiralis* ATCC 25486]	68

of acyl-CoA and GGSY$^{417-420}$ residues binding with carboxybiotin (Kiaptapan *et al*., 2001; Diacovich *et al*., 2004; Lin *et al*., 2006).

The *accA1* gene encodes a 74 kDa protein (673 amino acids, AccA1) which is for the α subunit of ACC (biotin carboxylase; BC). The predicted amino acid sequence for AccA1 also exhibited high similarity with other α subunits of ACCases from streptomycetes including *S. avermitilis*, *S. coelicolor*, *S. clavuligerus*, *S. griseus*, *S. pristinaespiralis*, *S. sviceus*, and *S.* sp. Mg1. The multiple sequence alignment of these sequences revealed that the ATP-binding motif (GGGKGG$^{162-167}$) and CO_2 fixation site (RECS$^{227-230}$) were present in AccA1. The conserved biotin-binding motif (MKM$^{629-631}$) in most biotin carboxylases was also found at the C-terminus (Kimura *et al.*, 2000).

Two other genes, *hmgL* encoding hydroxymethylglutaryl-CoA lyase and *fadST1* encoding acyl-CoA dehydrogenase were found in this gene locus. The FadST1 has been known to be a short chain acyl-CoA dehydrogenase that mediates α,β-dehydrogenation of the corresponding trans-enoyl-CoA. From the sequence analysis, it was assumed that this gene locus is mainly involved in the primary metabolism for the biosynthesis of fatty acids in *S. toxytricini*.

Subcloning, expression, and purification of AccA1 and BPL

The α subunit of ACCase (BC) has the biotin carboxyl carrier protein domain for biotin attachment and the biotin carboxylase domain for CO_2 fixation on the biotin moiety to form carboxybiotin-BC. Subsequently, the β subunit (CT) transfers the carboxyl group from biotin to acyl-CoA (Cronan and Waldrop, 2002).

In order to confirm the biological function of the cloned gene locus, the gene *accA1* was amplified and subcloned in a pET-28a(+) expression vector at *Bam*HI-*Hin*dIII sites. On the other hand, the gene *bpl*, identified in another cosmid clone (Demirev, 2009), was also cloned in the same expression vector at *Nde*I-*Xho*I sites. Two plasmids were transformed into *E. coli* BL21(DE3), and gene induction was performed with IPTG in LB broth. Both recombinant proteins exhibited significant solubility and subsequently were purified through Ni^{2+} affinity chromatography.

However *D*-biotin supplementation in culture medium caused a complete *in vivo* biotinylation of apo-AccA1 by the host *E. coli* BirA (a biotin-induced repressor protein A). Most of AccA1 was confirmed to be already biotinylated when non-biotinylated proteins (unbound fraction) were separated from the biotinylated ones (bound fraction) on the streptavidin beads (Fig. 3), implying that *D*-biotin induces host BirA production and facilitates the biotinylation of apo-AccA1. In addition, promiscuous activity of BirA capable of biotinylating other small proteins was observed.

In order to obtain non-biotinylated apo-AccA1, the protein was overproduced in LB medium without *D*-biotin (Fig. 3). The SDS-PAGE analysis showed that a small amount of holo-AccA1 was still present and that apo-AccA1 was not efficiently separated from other proteins. For *in vitro* biotinylation of apo-AccA1, the small amount of biotinylated AccA1 was removed by the streptavidin beads (Fig. 4).

In vitro biotinylation of recombinant apo-Acc1 by BPL

The separated apo-AccA1 was subjected to the *in vitro* biotinylation assay as an acceptor protein. After the reaction at 28°C, the biotinylated products were detected on 12% SDS-PAGE by HRP-conjugated streptavidin. The lower reaction temperature was attempted in order to present a culture condition of *S. toxytricini*. The analysis revealed that the biotinylation reaction occurred in a time-dependent manner within 2 h (Fig. 5). There were no positive signals in the control reactions, confirming the successful removal of holo-AccA1. Even though the *in vitro* biotinylation of apo-AccA1 by BPL had highest priority, a prolonged incubation resulted in non-specific biotinylation of other proteins as well, most likely due to the promiscuous activity of BPL (Choi-Rhee and Cronan, 2003; Choi-Rhee *et al*., 2004).

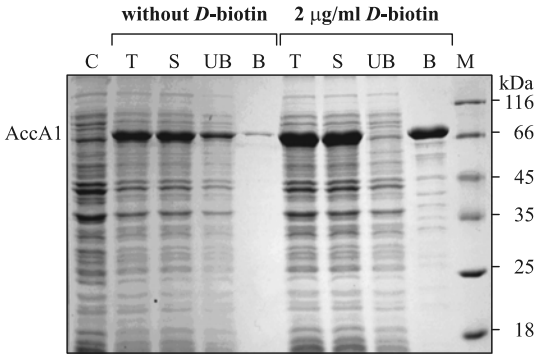

Fig. 3. Production of recombinant AccA1 in LB medium in the absence of *D*-biotin or with supplementation of *D*-biotin. Lanes: C, total cell protein without gene induction; T, total cell protein after gene induction; S, soluble protein fraction after lysis; UB, unbound fraction on the streptavidin beads; B, bound fraction on the streptavidin beads; M, protein marker.

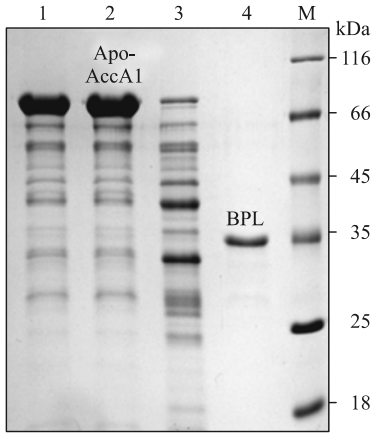

Fig. 4. Purified recombinant apo-AccA1 and BPL proteins. The biotinylated holo-AccA1 was removed from the recombinant protein using streptavidin beads. Lanes: 1, recombinant AccA1 purified by Ni^{2+} affinity column chromatography; 2, apo-AccA1 protein (unbound fraction on the streptavidin beads); 3, holo-AccA1 protein (bound fraction on the streptavidin beads); 4, recombinant BPL protein; M, protein marker.

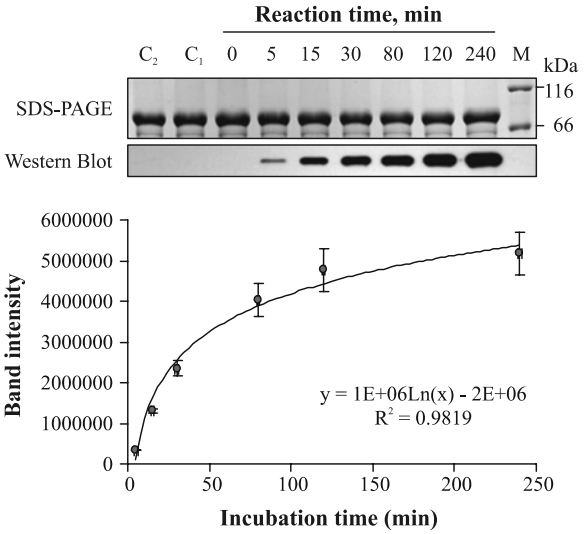

Fig. 5. *In vitro* biotinylation profile of apo-AccA1 depending on reaction time. The reaction was performed using a mixture of 1.25 μM of apo-AccA1, 70 nM of BPL, 0.03 mM *D*-biotin, 5.5 mM $MgCl_2$, 100 mM KCl, 5 μM dithiothreitol, and 30 mM ATP in Tris-HCl buffer (pH 7.9) at 28°C. C1, negative control without *D*-biotin; C2, negative control without BPL. The protein band intensities shown on the graph are Mean±SD of 3 independent experiments.

The *in vitro* biotinylation of apo-AccA1 with different concentrations of BPL was also examined. An increase of the BPL concentration led to a proportional rise in the amount of biotinylated holo-AccA1 (Fig. 6). Variations in KCl and NaCl concentrations did not alter the enzyme activity (data not shown).

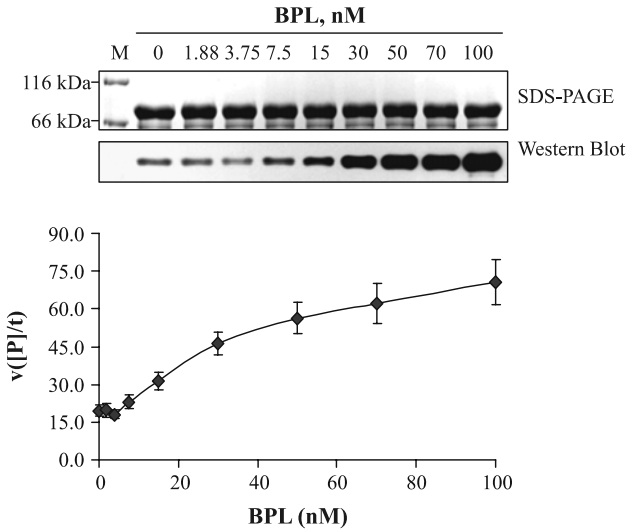

Fig. 6. *In vitro* biotinylation amount of apo-AccA1 by varying the concentration of BPL. The reaction was performed using a mixture of 1.25 μM of apo-AccA1, 0.03 mM *D*-biotin, 5.5 mM $MgCl_2$, 100 mM KCl, 5 μM dithiothreitol, and 30 mM ATP in Tris-HCl buffer (pH 7.9) at 28°C for 90 min with different concentration of BPL. The protein band intensities shown on the graph are Mean± SD of 3 independent experiments.

The successful *in vivo* and *in vitro* biotinylation of AccA1 suggests that the cloned *accA1* gene locus of *S. toxytricini* encodes a functional α subunit of ACC.

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