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ORIGINAL PAPER

SpnH from *Saccharopolyspora spinosa* encodes a rhamnosyl 4′-*O*-methyltransferase for biosynthesis of the insecticidal macrolide, spinosyn A

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Abstract Deoxysugar, 2′, 3′, 4′-tri-*O*-methylrhamnose is an essential structural component of spinosyn A and D, which are the active ingredients of the commercial insect control agent, Spinosad. The *spnH* gene, which was previously assigned as a rhamnose O-methyltransferase based on gene sequence homology, was cloned from the wild-type *Saccharopolyspora spinosa* and from a spinosyn K-producing mutant that was defective in the 4′-*O*-methylation of 2′, 3′-tri-*O*-methylrhamnose. DNA sequencing confirmed a mutation resulting in an amino acid substitution of G-165 to A-165 in the rhamnosyl 4′-*O*-methyltransferase of the mutant strain, and the subsequent sequence analysis showed that the mutation occurred in a highly conserved region of the translated amino acid sequence. Both *spnH* and the gene defective in 4′-*O*-methylation activity (*spnH165A*) were expressed heterologously in *E. coli* and were then purified to homogeneity using a His-tag affinity column. Substrate bioconversion studies showed that the enzyme encoded by *spnH*, but not *spnH165A*, could utilize spinosyn K as a substrate. When the wild-type *spnH* gene was transformed into the spinosyn K-producing mutant, spinosyn A production was restored. These results establish that the enzyme encoded by the *spnH* gene in wild-type *S. spinosa* is a rhamnosyl 4′-*O*-methyltransferase that is responsible for the final rhamnosyl methylation step in the biosynthesis of spinosyn A.

Keywords Spinosyn · *O*-methyltransferase · Polyketide · *Saccharopolyspora spinosa* · Insecticide

Introduction

Actinomycetes produce a great variety of bioactive compounds that have clinical, agricultural, and industrial applications, such as for antibiotics, suppression of fungal growth, control of parasites, preventing of the growth and proliferation of cancer and as insecticides. Many of these natural products are glycosylated and the biological activity is often correlated with the presence of sugars, such as 6-deoxyhexose, which contain many types of C- or O-methylation and transaminations.

A large number of hypothetical O-methyltransferases have been discovered in gene cluster cloning and genome-sequencing efforts, but only a small number O-methyltransferases from macrolide-producing organisms have been characterized by biochemical and genetic methods. For example, during the last two steps of tylosin biosynthesis, the intermediate demethylmacrocin is converted into macrocin, then to tylosin through two O-methylation steps by TylE and TylF O-methyltransferases [1, 2]. The *mycF* gene, which was cloned from *Micromonospora griseorubida,* was shown to encode for mycinamycin O-methyltransferase activity in *E. coli* [3]. The oleY gene from *Streptomyces antibioticus*, the oleandomycin producer, has been shown to catalyze the conversion of L-olivose into L-oleandrose

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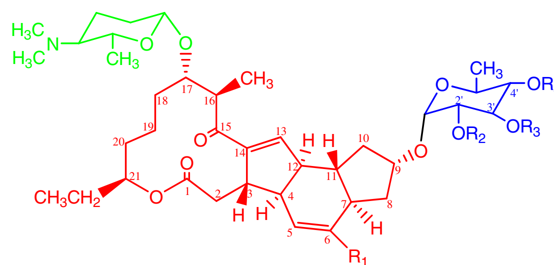
[4]. The *eryG* gene encodes for a protein that catalyzes the last step in the erythromycin biosynthesis in *Saccharopolyspora erythraea*, the 3-O-methylation of L-mycarose into L-cladinose [5]. The product from the *aveD* gene has been shown to catalyze the methylation of hydroxyl group at the C5 position of avermectin B in avermectin-producing *Streptomyces avermitilis*. This activity was confirmed through complementation of a mutant lacking avermectin B 5-O-methyltransferase activity with the wild-type strain [6]. Sequence analysis of the *aveD* region of the mutant strain revealed that a point mutation, Thr23 to Ile was within the ORF for the encoded protein. Patallo et al. [7] characterized three O-methyltransferases of antitumor elloramycin from *Streptomyces olivaceus*. In vivo and in vitro experiments showed that methyltransferases ElmI, ElmII, and ElMIII are responsible for the consecutive methylation of the hydroxyl groups at the 2′-, 3′-, and 4′-positions, respectively, after the sugar moiety has been attached to the aglycone.

Spinosyns, the active ingredients in Dow AgroSciences' commercial insecticides under the trademark Naturalyte®, are produced by fermentation of the actinomycete *Saccharopolyspora spinosa* [8]. Spinosyns are comprised of a 21-carbon tetracyclic macrolide containing forosamine and tri-*O*-methyl rhamnose with different degrees of methylation on the polyketide or deoxysugars. The most active components of spinosyn family compounds are spinosyn A and D that differ by a single methyl substitute at position 6 of polyketide (Fig. 1). Both the rhamnose and forosamine moieties are essential for the insecticidal activity of spinosyns [9].

The biosynthetic genes for spinosyn production include 19 genes on 74 kb of *S. spinosa* genomic DNA [10]. However, this gene cluster does not contain all of the genes required for synthesis and modification of the deoxysugars [11]. The spinosyn gene cluster contains five genes encoding a large polyketide synthetase (PKS), four genes involved in cross-bridging of polyketide lactone, two glycotransferases, five genes involved in forosamine biosynthesis, and three O-methyltransferase genes for rhamnose synthesis and modification. Because of the unique tetracyclic structure of spinosyns, the spinosyn genes have recently been the subjects of a number of investigations into the mechanisms of polyketide biosynthesis [12, 13].

Spinosyn biosynthetic studies indicated that the attachment of rhamnose to the aglycone is an obligatory first step in the conversion of the aglycone to spinosyns [14]. The three methylations are attached to rhamnose in a sequential order from the 2′-, 3′-, and 4′-hydroxyl groups. Three genes, *spnI*, *spnK* and *spnH* have been proposed to be responsible for these methylations. Due to the transcriptional effect on downstream genes, single cross-over knock out experiments could not make conclusive assignments between the gene products and the specific O-methyltransferase activity that is targeted at the 2′-, 3′-, or 4′-positions



Spinosyn	R1	R2	R3	R4
A	H	OCH3	OCH3	OCH3
D	CH3	OCH3	OCH3	OCH3
J	H	OCH3	OH	OCH3
H	H	OH	OCH3	OCH3
K	H	OCH3	OCH3	OH

Fig. 1 The structure of spinosyns. The dimethylamino sugar forosamine and tri-*O*-methylrhamnose are shown on the *left* and *right sides* of the tetracyclic aglycone, respectively

on the rhamnose sugar. Double cross-over genes knock out by using the appropriate resistance cassettes without transcriptional terminator or by making in-frame deletions could make conclusive assignments, but this method has not been successfully applied to *S. spinosa* yet.

In this report, we present biochemical and genetic evidence that the *spnH* gene from *S. spinosa* encodes a rhamnosyl 4′-O-methyltransferase that catalyzes the methylation of spinosyn K to form spinosyn A, which is the primary (~75%) active ingredient of the commercial insecticide, Spinosad.

Materials and methods

Microbial strains and growth conditions

Escherichia coli Topo10 (Invitrogen, Carlsbad, CA, USA) and BL21(DE3), BL21(DE3) pLysS and AD494 (EMD Biosciences, Gibbstown, NJ, USA) used for DNA cloning and protein expression were grown on LB agar (BD, Franklin Lakes, NJ, USA). Apramycin and kanamycin (Sigma Chemical Co., St Louis, MO, USA) were added to LB at 50 mg/l. *Saccharopolyspora spinosa* NRRL 18539 was a re-isolated strain from originally isolated *S. spinosa* strain. *S. spinosa* NRRL 18743 were derived from *S. spinosa* NRRL 18539 through mutagenesis. For genomic DNA iso-

lation, *S. spinosa* NRRL 18539 and NRRL 18743 were grown in INV-2 media (9.0 g/l dextrose, 30 g/L tryptiase soy broth, 3.0 g/L yeast extract, 2.0 g/L magnesium sulfate), and for fermentation, *S. spinosa* or derivative cultures were grown, extracted and analyzed by LC/MS according to Zahn et al. [15].

Molecular methods

Unless specifically listed, standard protocols for DNA manipulations were used [16]. Chromosomal DNA was isolated using a Genomic DNA purification kit (Qiagen Inc., Valencia, CA, USA) and plasmid DNA was isolated using the NucleoSpin Nucleic Acid Purification Kit (CLONTECH Laboratories, Inc., Palo Alto, CA, USA). *SpnH* and its mutant genes were PCR amplified using AmpliTaq Polymerase Kit (Perkin Elmer/Roche, Branchburg, NJ, USA) in a 48-sample DNA Thermal Cycler (Perkin Elmer Cetus) under the following conditions: (1) 94 °C, 2 min, (2) 94 °C 30 s, 58 °C 1 min, 72 °C 2 min, 25 cycles and (3) 72 °C 10 min; 1 cycle. PCR products were gel-extracted utilizing Qiagen II Gel extraction Kit and then cloned into TA vector (Invitrogen). DNA sequencing was confirmed by fluorescent cycle sequencing according to the methods of Burgett and Rosteck [17] under thermal cycler conditions: 96 °C, 30 s, 50 °C, 20 s, 60 °C 4 min; 25 cycles with a 377 ABI Prism Sequencer (Applied Biosystems, Inc., Foster City, CA, USA). For construction of plasmid pSE152/SpnGH, spnGH and the promoter region were amplified by PCR with primers spnGHF 5′-CCGgAATTCCCTgATCTCTg CAACgTT CACCTTC-3′, which had a *EcoR* I site (underline) at its 5′ end, and primer spnGHR 5′-CTA gCgTCTA gATCACCAgCTgCggCgCCAgTAggC-3′, which had a *Xba*I site (underline) at its 5′ end. The amplified DNA fragment was digested with *EcoR* I and *Xba*I, and then cloned into pSET152 at the same cloning sites to form pSET152/spnGH.

Transformation of *S. spinosa*

Plasmid pSET152/SpnGH was transferred from *E. coli* ATCC 47055 into *S. spinosa* NRRL 18743 by conjugal transfer [18].

Expression and purification of spnH and spnH165A in *E. coli*

The *spnH* and *spnH165A* genes were amplified by PCR with primer spnHF 5′-GGGAATTCCATATGCCCTCCC AGAACGCGCTGTAC-3′, which had an *Nde*I site (underline) at its 5′ end, and primer spnHR 5′-GCCGCTCGAG CCAGCTGCGGCGCCAGTAGGCGCCCGT-3′, which had an *Xho*I site (underline) at its 5′ end. The genomic DNA of *S. spinosa* NRRP 18539 and NRRL 18743 was prepared and used as a template for PCR. The amplified gene was inserted into *Nde*I and *Xho*I sites of the vector pET26b (EMD Biosciences) to generate pET*spnH* and pET*spnH165A*, respectively. The *spnH* and *spnH165* that were expressed by these vectors were fused with a C-terminal His tag. *E. coli* BL21(DE3) (EMD Biosciences) was then transformed with pET*spnH* and pET*spnH165A*. Recombinant strains were cultivated in Luria–Bertain medium supplemented with kanamycin (50 µg/ml). After induction with 1 mM iso-propyl-β-D-thiogalactopyranoside (IPTG) at 37 °C, a significant fraction of the heterologously expressed proteins from *spnH* and *spnH165A* were found to be present as inclusion bodies in the fermentation broth.

Purification of recombinant spnH or spnH165A was performed according to the procedure described in the Talon metal affinity resin manual (Clontech, CA). The purification data were based on a 500-ml culture. Protein concentrations were determined by the method of Bradford using the Bio-Rad protein assay kit and bovine serum albumin as standard. SDS-PAGE gel (10%) was carried out according to the modified procedure described by Laemmli [19]. After electrophoresis, gels were stained with Coomassie brilliant blue.

Cultures of *E. coli* BL21 (DE3)/pETSpnH or pETSpnH165A were harvested at 4 h after IPTG (1 mM) induction by centrifugation for 10 min at 4,000*g*. The pellet from 500-ml culture was resuspended in 20 ml lysis buffer containing 100 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM PMSF, 1 mM $MgCl_2$. The cells were lysed by sonication at full power four times on ice while maintaining the temperature below 8 °C. The inclusion bodies were collected by centrifuge at 12,000*g* for 15 min and then washed twice with lysis buffer containing 3% (w/v) sodium dexoycholate. The washed inclusion bodies were collected by centrifugation at 25,000*g* for 10 min and then extracted with 50 ml of lysis buffer containing 0.5 M urea. Undissolved pellet residue was removed by centrifugation. The clarified extract was mixed with Talon metal affinity resin and the mixture was gently agitated at 4 °C for 30 min and then centrifuged at 700*g* for 5 min. The supernatant was discarded and the resin washed four times with 30 ml of lysis buffer. The washed resin was then resuspended in lysis buffer and transferred to the gravity column. The column was washed first with 15 ml lysis buffer. Subsequently, the spnH or spnH165A was eluted with 20 ml lysis buffer containing 50 mM imidazole and 0.1 M NaCl. The eluted fractions were combined and dialysed overnight against 50 mM Tris–HCl, pH 8.0, 1 mM DDT, 10% glycerol and then stored at 70 °C until used.

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SPNH165A      1 -------------------------MPSQNALYLDLLKKVLTNTIYSD-RPHPNAWQDNTDYRQAARAKGTDWPTVAHTM
SPNH          1 -------------------------MPSQNALYLDLLKKVLTNTIYSD-RPHPNAWQDNTDYRQAARAKGTDWPTVAHTM
ELMIII        1 ------------------------MTEDARDLYLDLMKKVLTNLIYRD-APIQTFVYD-GEPDADPRLLGRDWPSVAHTM
CLOP          1 MEVTPIVVSVADNSLQAQPEGPTQGTSSGSWLYLNLMKKVLAGTVYEDPAHRQNFSHDDATYREEVRNEGRDWPANAHTM
COUP          1 -MEVAPIVSRVAHKLRSQPEETTQGTGSDSSLYLDLMIKVLAGTVYEDPAHRENFSHRDSTYREEVRSEGRDWPANAHTM
TYLF          1 ---------------------MAPSPDHARDLYIELLKKVVSNVIYEDPTHVAGMITD-ASFDRTSRESGEDYPTVAHTM
MYCF          1 -----------------------MSPSTGVELYLDLLKRTVSNFIYQDATHVAGLITE-AAFVEEARESGEDYPTVAHTM
CHMII         1 ----------------------MTVIAAEADLYLDLLKKVVTNMIYEDQTNVAGLITS-SSYSAELRSVGEDFPRVAHSM
consensus     1                                LYl Ll k l   iY D                  R  G DwP  AHtM

SPNH165      55 IGLERLDNLQHCVEAVLADGVPGDFAETGVWRGGACIFMRAVLQAFGDTGRTVWVVDSFQGMPESSAQDHQADQAMALHE
SPNH         55 IGLERLDNLQHCVEAVLADGVPGDFAETGVWRGGACIFMRAVLQAFGDTGRTVWVVDSFQGMPESSAQDHQADQAMALHE
ELMIII       55 VGLKRLDNLQYCVETVLADGVPGDLVETGVWRGGSSIFMRAVLRAHGDTARRVWVADSFEGMPEVGADSHAVDREMRLHE
CLOP         81 IGIKRLENIQQCVEDVIGNNIPGDLAETGVWRGGACIFMRGILRAHDVRDRTVWVADSFQGIPDVGADGYPGDRRMALHR
COUP         80 IGIKRLENIRQCVEDVIGNNVPGDLVETGVWRGGACIFMRGILRAHDVRDRTVWVADSFQGIPDVGEDGHAGDRKMALHR
TYLF         59 IGLKRLDNLHRCLADVVEDGVPGDFIETGVWRGGACIFARGLLNAYGQADRTVWVADSFQGFPELTGSDHPLDVEIDLHQ
MYCF         57 IGMKRLNNLQHCVESALRDGVPGDVLETGVWRGGACIFARGILKAYDVRDRTVWVADSFQGFPKITDDDHPMDAEMNLHQ
CHMII        58 VGLKRLDNLQKCLEDVLRDGVPGDFAETGVWRGGACIFARGVFRAHGVRDRKVWVADSFQGFPKTTEDDHQLDVDIDLGQ
consensus    81 iGl RL Nl  Cv   l   vPGD  ETGVWRGG  IF Rav  A     R VWV DSF G P         D  m L
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SPNH165A    135 YNDVLGVSL--ETVRQNFARYGLLDEQVRFLPAWFRDTLPTAPIQELAVLRLDGDLYESTMDSLRNLYPKLSPGGFVIID
SPNH        135 YNDVLGVSL--ETVRQNFARYGLLDEQVRFLPGWFRDTLPTAPIQELAVLRLDGDLYESTMDSLRNLYPKLSPGGFVIID
ELMIII      135 HNGVLAVPL--EQVRANFERYGLLDDQVRFLPGWFKDTLPGAPTGRLAVIRLDGDLYESTTDALENLMPRLSPGGFVIID
CLOP        161 HNAVLAVSE--DEVRRNFRNYDLLDEQVRFLPGWFKDTLPTAPIDALAVLRMDGDLYESTWDTLTNLYPKVSVGGYVIVD
COUP        160 RNAVLAVSE--DEVRRNFRNYDLLDEQVRFLPGWFKDTLPTAPIDALAVLRMDGDLYESTWDTLTNLYPKVSVGGYVIVD
TYLF        139 YNEAVDLPTSEETVRENFARYGLLDDNVRFLAGWFKDTMPAAPVKQLAVMRLDGDSYGATMDVLDSLYERLSPGGYVIVD
MYCF        137 YNEAVDLPTSLATVQRNFSRYGLLDDQVRFLPGWFKDTMPTAPFERLAVLRMDGDSYGATMDVLTHAYPRLSPGGFAIID
CHMII       138 YNDVLSIPVDVETVKGNFARYGLLDDQVRFLPGWFKDTMPTAPIERLAVLRLDGDSYAATREVLTNLYHKVSDGGYVIVD
consensus   161  N  l v      V  NF  Y LLDeqVRFL GWFrDTlP AP   LAVlRlDGD Y  T d L     klS GGf IiD

SPNH165A    213 DYFLP-SCQDAVKGFRAELGITEPIHDIDGTCAYWRRSW-----------------------------------------
SPNH        213 DYFLP-SCQDAVKGFRAELGITEPIHDIDGTCAYWRRSW-----------------------------------------
ELMIII      213 DYAID-ACRDAVHDYRGRYGISDPISEIDGTGVFWRHTAASARSLQPATV------------------------------
CLOP        239 DYLMCPPCKDAVDEYRARFDIADELIRIDRDGVYWQRTR-----------------------------------------
COUP        238 DYLMCPPCKDAVDEYRARFGIADELIRIDRDGVYWQRTE-----------------------------------------
TYLF        219 DYCIP-ACREAVHDFRDRLGIRDTIHRIDRQGAYWRHSG-----------------------------------------
MYCF        217 DYCIP-ACREAVHEYRDRHGISDEIVEIDRQGVYWRSLGLTFPVHCRRSGRSGPTTGACHHTTGSSSGPRSNIPSLHRSC
CHMII       218 DYCIP-ACRQAVHEFRDEHGITDEIHQIDRQGSYWRRSS-----------------------------------------
consensus   241 DY l   C  AV  fR    I e i  ID  G yW

SPNH165A        --------
SPNH            --------
ELMIII          --------
CLOP            --------
COUP            --------
TYLF            --------
MYCF        296 VSGIPSRI
CHMII           --------
consensus
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Fig. 2 Alignment of the deduced amino acid sequences of spnH and spnH165A with different O-methyltransferases. TYLF (*AAD41819.1*) from *Streptomyces fradiae* [5, 6]; MYCF(*BAA03670.1*) from *S. mycarofaciens* [7]; ELMIII (*CAD57141.1*) from *S. olivaceus* [8]; COUP (*AAG29794.1*) from *S. rishiriensis*; CLOP (*AAN65238.1*) from *S. roseochromogenes* subsp. *Oscitans*; CHMII (*AAS79452.1*) from *S. bikiniensis*. The *spnH* and *spnH165A* genes are described in this paper. The *arrow* indicates the mutation

Assay of spnH and spnH165A

Heterologously expressed pure proteins of spnH and spnH165A were incubated in a reaction mixture containing 100 mg/L of purified spinosyn (J, K, or H), 0.1 μmol S-adenosyl-methionine, 10 μmol of $MgSO_4$ in 0.5 ml of 0.1 M phosphate buffer (pH 7.8) for 30 min at 30 °C, and the filtered methanolic extracts were analyzed by liquid chromatography-positive-ion ESI-mass spectrometry [15, 20].

Results

Sequence analysis of the *spnH* gene from wild type and spinosyn K mutant strains

The spinosyn K mutant (NRRL18743) used in this study was obtained through random mutagenesis of the wild type *S. spinosa* strain (NRRL18539) using the mutagen, *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (NTG) [21]. The defective *spnH* gene from the spinosyn K mutant strain was amplified by polymerase chain reaction (PCR) from genomic DNA, and then was cloned into the TA vector (Invitrogen). Analysis of the DNA sequences identified a single base substitution from G to A that occurred at position 424 bp that resulted in a change in the translated amino acid sequence from Gly (G) to Asp (A) at amino acid position 165 (Fig. 2). A sequence comparison of the wild-type *spnH*, the defective *spnH* gene from the spinosyn K mutant (referred to *spnH165A*), and related O-methyltansferases from other bacterial species revealed that the amino acid substitution in *spnH165A* occurred in a highly conserved region of the translated amino acid sequence. The location of this mutation provided initial evidence that the defect in 4′-*O*-methyltransferase activity was directly caused by the G to A substitution (Fig. 2).

Expression and Purification of *spnH* and *spnH165A* by *E. coli*

The level of expression and the solubility were examined in different *E. coli* strains BL21 (DE3), BL21 (DE3) pLysS, AD494, and no significant difference was found among these strains; the protein spnH or spnH165A was expressed in inclusion bodies. Overexpression was induced by varying the concentration of IPTG; 1 mM IPTG was the best induction concentration for spnH or spnH165A synthesis. Low concentration of urea was used to refolded spnH or spnH165A from inclusion bodies; this method has also been applied to purify other proteins [22]. One step His tag affinity chromatography was able to purify spnH or spnH165A protein (Fig. 3).

The apparent molecular weight of the major polypeptide present in each broth sample, as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was 30 kDa (Fig. 3), which was in good agreement with the translated amino acid sequence of the *spnH* and *spnH165A*. Densitometry of the protein bands in the gel showed that the purity of the 30-kDa band was more than 95%. The typical yield of the heterologously expressed proteins from pETSpnH and pETSpnH165A was 3 mg of the purified protein per liter of *E. coli* broth. The N-terminal amino acid sequence analysis of *spnH* and *spnH165A* showed that both polypeptides lacked an amino-terminal methionine residue, which is not uncommon considering that N-terminal methionine could be cleaved by methionine aminopeptidases in both prokaryotic and eukaryotic systems [23].

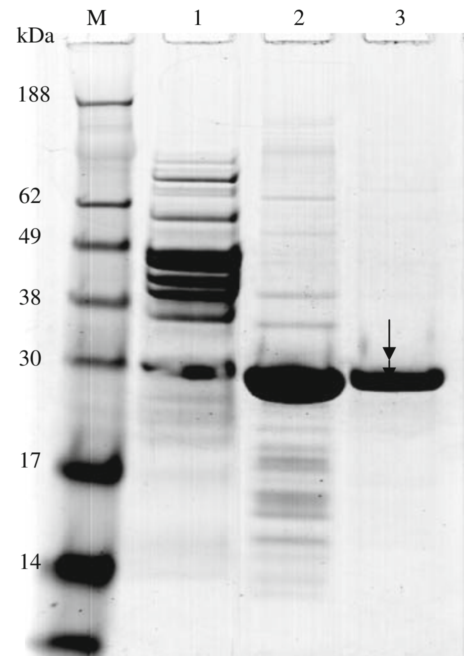

Fig. 3 SDS-PAGE analysis of proteins heterologously expressed in *E. coli*. *Lane M* protein molecular weight standard (invitrogen), *lane 1* proteins extracted from whole cells following an IPTG induction period of 4 h, *lane 2* protein extracted from inclusion bodies with 0.5 M urea, *lane 4* His-tag purified *spnH*

In vitro enzymatic assay of *spnH* and *spnH165A* activity

In order to determine the putative substrates of the *spnH* gene product, we established an in vitro methylation assay to test the 4′-O-methyltransferase activity of wild-type and spinosyn K-mutant strain of *S. spinosa*. The in vitro assay utilized purified spinosyns J, K, and H as substrates (Fig. 1). The LC-MS result showed that the protein product from the wild-type *spnH* gene was capable of methylating spinosyn K (Fig. 4, panel 2), but could not methylate spinosyn J, or H (data not shown). The gene product from *spnH165A* showed no activity in the presence of spinosyns K (Fig. 4, panel 3), J, or H (data not shown). This result indicated that the single mutation from G-165 to A-165 caused the disruption in *spnH* activity. These results also showed that the enzyme specificity of *spnH* for its substrate, spinosyn K, was high and that methylation was dependent upon the presence of exogenously supplied S-adenosyl-methionine, indicating that the enzyme encoded by *spnH* is an S-adenosyl-methionine-dependent methyltransferase. This result is consistent with previously published ^{13}C-labeling experiments, which showed that the two

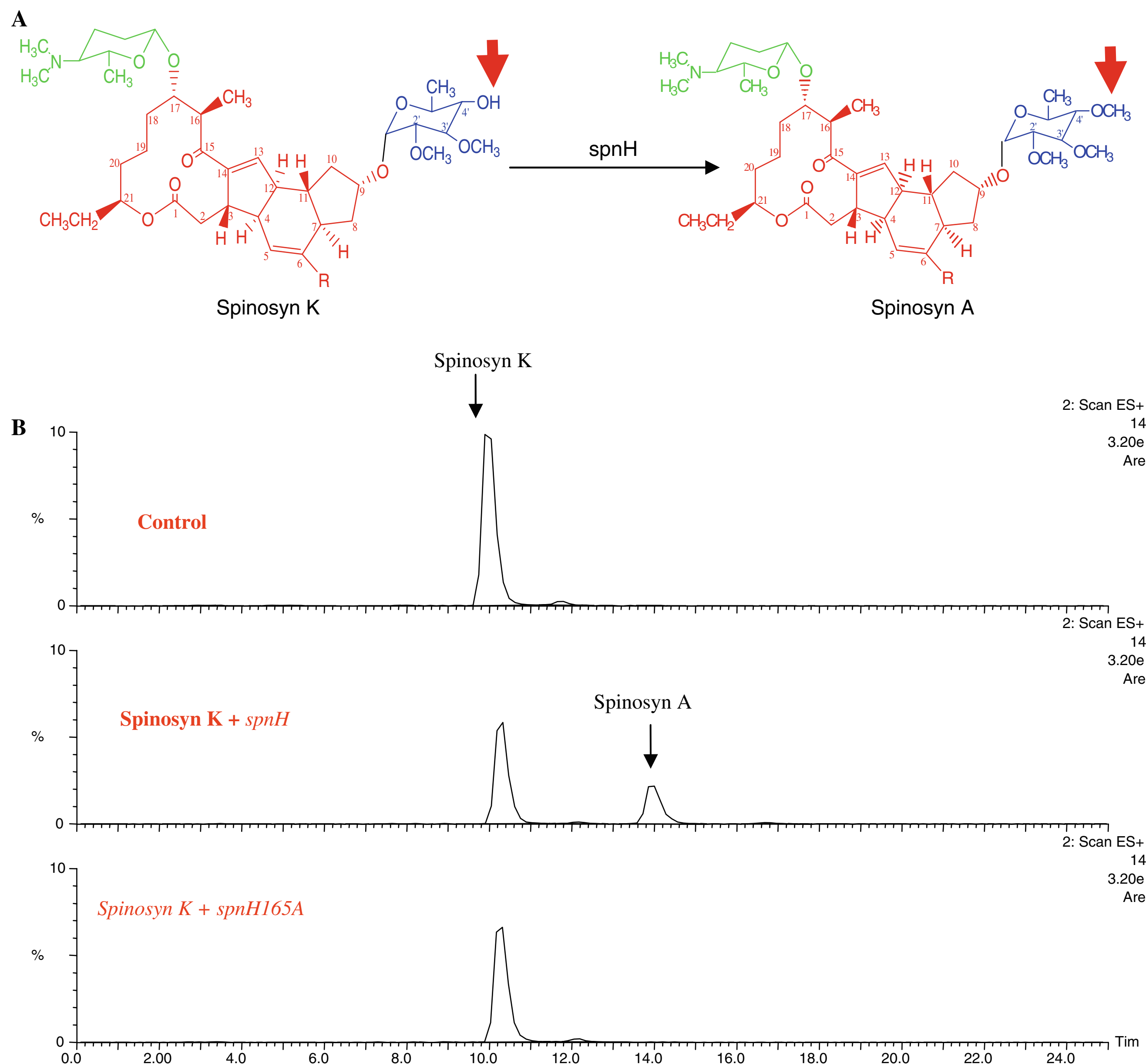

Fig. 4 In vitro assay of spnH. **a** The rhamnosyl 4′-O-methyltransferase reaction catalyzed by the *spnH* gene product. **b** LC-MS analyses of enzymatic reaction mixtures using spinosyn K and the gene products from *spnH* and *spnH165A*

N-methyl groups of forosamine, and three O-methyl groups of 2′, 3′, 4′-tri-*O*-methylrhamnose, were derived from S-adenosyl-methionine [14].

In vivo assay of spnH activity

In order to confirm the specific function of *spnH*, the *spnH* gene was transformed into the wild type and spinosyn K mutant strains containing the defective *spnH165A* gene. The goal of this experiment was to verify whether the *spnH* gene from the wild-type *S. spinosa* could restore the 4′-O-methylation activity of 2′, 3′, and 4′-tri-*O*-methylrhamnose in the mutant strain. The *spnH* and the adjoining *spnG* genes were cloned into the plasmid pSET152 and then transformed into the *S. spinosa* spinosyn K mutant through conjugation [18]. The transformants were fermented in shake flasks according to the procedure described previously [24], and the filtered methanolic extracts were analyzed by LC-MS [20]. The total ion current chromatograms for the LC-MS analysis showed that the *S. spinosa* mutant that contained the defective *spnH165A* gene produced spinosyn K, but no detectable levels of spinosyn A (data not shown). The 4′-O-methylation activity for 2′, 3′-tri-*O*-methylrhamnose was restored in the transformed mutant strain, which contained copies of the *spnH* and *spnG* from the wild-type *S. spinosa*.

Discussion

In the biosynthetic pathway of spinosyn, three O-methyltransferases (*spnI*, *spnK* and *spnH*) produced a 2′, 3′, 4′-tri-*O*-methylated rhamnose by successive S-adenosyl-methionine-dependent methylation reactions. Permethylated L-rhamnose is unusual in its highly unreactive chemical nature, and its infrequent occurrence in nature. This completely O-methylated sugar has been reported only two other times in nature: (a) in the antitumor polyketide, elloramycin, which is produced by *S. olivacus* [7] and (b) in the structurally similar macrolide, butenyl-spinosyn, which is produced by *Saccharopolyspora pogona* [25]. The initial functional assignment of *spnI*, *spnK* and *spnH* to the O-methyltransferase family came from sequence homology comparisons to other established methyltransferases from antibiotic-producing microorganisms [5, 15]. In this study, the in vivo and in vitro results have established that *spnH* encodes for a 4′-O-methyltransferase that is responsible for the final rhamnosyl methylation step in the biosynthesis of spinosyn A. The data also show that a single amino acid substitution of G-165 to A-165 in a spinosyn K mutant strain fully abolishes the rhamnosyl 4′-O-methyltransferase activity of the heterologously expressed *spnH165A* gene product. The inability of wild-type *spnH* gene product to methylate spinosyns J, and K indicates that the enzyme is highly specific for the substrate, 2′, and 3′-tri-*O*-methylrhamnose. This result suggests that the order of the O-methylation is an important factor in the activity of these enzymes. In the elloramycin biosynthesis pathway, three O-methyltransferases have been shown to be responsible for the consecutive methylation of hydroxyl groups at the 2′-, 3′-, and 4′-position, respectively, after the rhamnose moiety has been attached to the aglycone [7]. In *S. spinosa*, it has not yet been determined whether the methylations normally occur before or after the attachment of the rhamnose moiety to the aglycone.

Future work on the spinosyn biosynthesis pathway will be targeted at elucidating the functions of two other O-methyltransferases, *spnK* and *spnI*, and further characterizing the substrate specificity and catalytic properties of these enzymes. Plant-derived O-methyltransferases [26], tcmO from the tetracenomycin biosynthetic pathway of *S. glaucescens* [27], and the 7-O-methyltransferase from *S. avermilitis* have been shown to exhibit a high degree of promiscuity in substrate binding and catalysis of sugars, and therefore, have been proposed to be suitable biocatalysts for increasing chemical diversity from combinatorial chemistry programs. Similarly a better understanding of substrate binding and catalytic properties of the O-methyltransferases (*spnI*, *spnK* and *spnH*) from *S. spinosa* may facilitate future applications of these enzymes in combinatorial biosynthesis.

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