University of Nevada, Reno

Functional Characterization of Fatty Acyl-Reductase CG17560 in Drosophila Melanogaster

A thesis submitted in partial fulfillment
of the requirements for the degree of

BACHELORS OF SCIENCE
IN BIOTECHNOLOGY

by

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May, 2014

Abstract

Over the past few decades, there has been growing interest in biofuel development to supplement current energy sources, with different approaches being explored. Use of enzymes from insects, such as fatty-acyl reductases (FARs), has been one such approach. FARs convert fatty acids to equivalent aldehydes and/or alcohols. Aldehydes may serve as precursors for decarbonylation, resulting in hydrocarbon production which could then be purified as biofuel. The FAR 17560 protein from *Drosophila melanogaster* was analyzed for its substrate specificity and resulting product. The experiment involved baculoviral expression of the FAR 17560 gene in SF9 cells and functional assays with various free fatty acid substrates, using whole cell incubations. Initial reactions with infected cells producing recombinant FAR 17560 showed uptake of substrate when given a free fatty acid cocktail (24:0, 26:0, 28:0) with only 24:0 being taken up in preferentially, however aldehyde or alcohol products from these substrates were not detected . The free fatty acid 18:0 was also tested for substrate specificity. These data suggest that FAR 17560 may have specificity for 24:0 FFA and smaller chain length FFAs. This suggests that FARs work together with other enzymes not present in the SF9 cells, which would explain why only FFA uptake without substrate modification was observed. As such, conducting additional research can help to further identify the specificity of FAR 17560 protein.

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Introduction

Due to increasing energy consumption for transportation, industry, commerce, and residential sectors, current energy sources such as petroleum are strained to meet today's rising demands; a problem for which alternative energy could be a potential solution. As of 2009, petroleum-derived fuels are the primary energy source making up 35% of total energy sources available with natural gas and coal making up 23% and 19%, respectively, with reserves decreasing rapidly (QTR 2011). The benefits of alternative or non-crude-derived liquid fuels include decreased greenhouse gases and particles which lead to reduced haze and better air quality. Ethanol is the most common form of alternative energy derived from biomass with Brazil and the U.S. leading in production rates: roughly 22.5 billion gallons of ethanol was produced globally in 2010, making up about 10% of motor gasoline consumption by volume (QTR 2012). The rising interest in biofuels is especially evident in the United States. There was an 800% increase in biofuel production between the years of 2000 to 2010, which equated to 13 billion gallons of ethanol from corn grain alone (QTR 2012). The attractiveness of plant-based approaches using corn, sugarcane or algae where benefits include lower greenhouse gas (GHG) emissions and renewability. Some considerations, however, include the effects of increasing biofuel to food crop ratios, farmland requirements, and the large amount of fuel required for maintenance.

There are many ways to produce biofuel with some involving the biosynthetic conversion of long-chain fatty acids (LCFA) to hydrocarbons via cyanobacteria, plants, and insects. While all three use LCFA, there are some distinct differences during the conversion processes as well as the enzymes involved. One method of hydrocarbon

production uses cyanobacteria, also known as blue-green algae, which can take up the LCFA for conjugation to an acyl carrier protein (ACP) and convert it to a fatty aldehyde. The aldehyde undergoes decarbonylation (*n*-1 model) by a cytochrome P450 with both high sequence and functional similarity to ω-oxidases resulting in olefin hydrocarbon production (Schirmer et al. 2010). A second method of hydrocarbon production involves the natural hydrocarbon-producing mechanisms in plants. Many plants produce cuticular waxes as a natural defense against sun and insect damage and to retain moisture. The waxes can range from C_{20}-C_{34} straight chain aliphatics which are derived from very-long-chain fatty acids (VLCFA), a mechanism which suggests a possible route to high energy biofuels (Jetter et al. 2008). The known mechanism starts with the *de novo* fatty acid synthesis catalyzed by fatty acid synthase (FAS) and a series of four reactions with intermediates attached to acyl-carrier proteins (ACP) for chain elongation by fatty chain elongases (FAE). To reach elongation sites, fatty acid is hydrolyzed from ACP to CoA. Finally, the elongated fatty acyl-CoA gets reductively decarbonylated to produce hydrocarbons, possibly by CER1 (Bourdenx et al. 2011) The third method uses enzymes found in insects. Insects produce hydrocarbons as a waxy cuticular layer to prevent desiccation and for chemical communication. Insect hydrocarbon biosynthesis involves reduction of fatty acyl-CoA to fatty aldehyde, which is then oxidized to hydrocarbon via oxidative decarbonylation by CYP4G2 (Y. Qiu et al. 2012). However, an aldehyde-producing FAR has not been characterized yet for any insect.

FAR 17560 from *Drosophila melanogaster* was tested as a possible candidate for aldehyde production. Promising results after treatment with free fatty acid substrates and

a modified functional assay using the whole cell method may provide insights to its mechanism as well as lead to a possible biofuel source. It is hypothesized that when provided with free fatty acid substrate, CG17560 would yield aldehyde products readily available for CYP4G2 uptake. The use of the whole cell method was expected to prevent complications arising from protein preparation that could potentially lead to CG17560 deactivation.

Methods

Transfer to competent cells

Drosophila melanogaster fatty acyl-CoA reductase cDNA clones for CG17560 were purchased from the Drosophila Genomics Center (DGRC). The FAR 17560 clones were sampled from the Whatman disks using the protocol included with the DGRC purchase, with some modifications. Fifty µl of sterile 1X TE was added to the Whatman disk and was rinsed briefly by pipetting up and down twice before removing the TE. The tube was incubated on ice while 50 µl of Stellar™ Competent Cells (Clonetech, Mountainview, CA) were added and incubated on ice for 30 min. Cells were vortexed briefly and after 35 min, heat shocked at 37°C for 2 min, then placed back on ice for an additional 2 min. The cells were transferred off the disk to 1 mL SOC media instead of the recommended LB. The cell/media mixture was incubated at 37°C for 1 h with shaking with proper aeration. 50, 100 and 200 µl aliquots of the transformation were plated on LB chloramphenicol (20 µg/ml) plates and incubated overnight at 37°C and remaining transformations were stored at 4°C.

Colony Verification

Seven colonies were selected from replica plates to confirm the CG17560 insert into POT2 by standard PCR screening with vector primers, followed by plasmid preparation using Qiaprep Spin Miniprep Kit and sequencing using the vector and gene specific primers indicated in Table 1 (CG17560). All oligonucleotide primers were synthesized and purchased from either Integrated DNA technologies or Sigma Aldrich.

Expression Cloning

FAR 17560, cDNA was subcloned into the pENTRNco1- modified GateWay plasmid. The FAR insert was modified for a Gibson assembly reaction by using specific primers (CG17560 InFusF and CG17560 InFusR; Table 1) designed to add 3' and 5' overhangs on the fragments that enabled cloning into the target vector, pENTRNco1-. Using purified plasmid DNA as the template, 1 µl of template was combined with 5 µl 10X PFU ultra II reaction buffer 2.5 mM MgCl2, 0.1 mM dNTP, 0.4 pM forward and reverse primers, 0.025 U/ µl PFU Ultra II HS DNA polymerase and 40.5 µl sterile H_2O for a final reaction volume of 50 µl. Cycling parameters were as follows: 95°C for 1 min, 94°C for 40 s, 56°C for 1 min 30 s, 0.3°/s increase to 72°C, 72°C for 1 min 30 s. Steps 2-5 were repeated 2 times, then 94°C for 40 s, 67°C for 30 s, 72°C for 1 min 30 s. Steps 7-9 were repeated 34 times and the final annealing time was 72°C for 6 min with a final hold at 4°C.

Products from PCR reactions were purified from 1% agarose gels using the Illustra GFX PCR DNA and Gel Band purification kit and followed the provided protocol.

Linearized pENTRNco-1 DNA (Sandstrom et al, 2006) was prepared in50 µl reactions containing: 5 µl 10X PFU ultra II reaction buffer, 2.5mM MgCl2, 0.1 mM dNTP, 0.4 pM forward and reverse primers (pENTR F4/pENTR R5), 0.0.25 U /µl PFU Ultra II HS DNA polymerase and 40.5 µl sterile H2O. Cycling parameters: 95°C for 1 min, 94°C for 40 s, 67°C for 30 s, 72°C for 45 s. Steps 2-4 were repeated 30 times, then 72°C for 3 min and set to a final hold at 4°C.

Gibson assembly of insert and plasmid DNA (total of 20 µl CG17560) was performed by mixing 1.5x the amount of linearized plasmid (pENTR-NCO) and 10 µl of Buffer 4 and 1 µl of Dpn1. The reaction was made up to a total volume of 100 µl with sterile H2O and incubated at 37°C for 1 h. Digested products were purified using the Nucleospin gel and PCR Clean-up kit as per the manufacturer's instructions. Purified insert and vector infusion reaction was completed using the Eco Dry Kit which had the protocol as follows: 10 µl of linearized vector and insert in H2O was added to the Eco Dry pellet and pipetted to mix before incubating at 37°C for 15 min. and then 50°C for 15 min.

Transformation of CG17560 into Competent Cells

A total of 2.5 µl of the CG17560 infusion reaction was added to 1 tube of thawed *E. coli* Stellar™ Competent Cells for transformation and incubated on ice for 45 min. They were then transformed into E. coli Stellar Competent cells by standard methods. Transformants were selected following overnight growth on LB-kanamycin plates at 37 °C. Recombinant colonies were identified using the colony PCR protocol and the integrity of the inserts was confirmed by sequencing using pENTRF2/pENTRR2 vector primers and gene specific primers (Table 1).

Sequencing

Liquid culture of CG17560 colony 1 was purified with the Nucleospin kit and submitted for full length sequencing. All sequencing reactions were carried out by the

Nevada Genomics Center (Reno, NV.), using dideoxy sequencing on an ABI 3700 sequencer and analyzed using Vector NTI (Version 9.0.0) software.

Transfection and confirmation of pENTR constructs into Baculovirus

LR recombination reaction was used to transfer pENTR insert DNA into Baculo-Direct Baculoviral DNA (Invitrogen) and confirmed by PCR before proceeding to infection. 10 uL of Bac Linear DNA was added to 2 uL of 17560 pENTR ppDNA from colony 1, 4 uL of TE, and 4 uL of Clonase II before incubating overnight at 25°C. Infection of Sf9 cells was performed following the manufacturer's instructions with the addition of 120 U/µl of penicillin and streptomycin, 12 µl of 100mM Gancyclovir and 12 µl of Fungizone. P1 viral stocks were harvested by centrifugation in Beckman GS-6R centrifuge at 3000 RPM (4°C) for 10 min and collecting of the supernatant. The presence of insert DNA in the first viral stock (P1) was confirmed by PCR using the primers Bac F1 and Bac R2. P2 and P3 viral stocks were produced by successive amplifications according to the manufacturer's instructions, and the integrity of the insert was re-confirmed by sequencing PCR products produced by amplifications of isolated viral DNA using Bac F1 and Bac R2 as well as the appropriate gene specific primers 17560 F2 and 17560 F3. Baculoviral DNA was isolated using the MasterPure complete DNA and RNA purification kit according to the manufacturer's instructions. 1:100 dilutions of purified viral DNA were used as template for PCR confirmation of P1, P2 and P3 viral stocks at the Genomics Center.

Plaque assay

A plaque assay was performed according to the Baculovirus protocol to measure the titer of the P3 viral stock. 0.4 x 10^6 sf9 cells were seeded per well, 2 mL total for each, and left to incubate for 1 hour at 27°C. Viral dilutions from 10^{-5} to 10^{-9} were prepared and added to wells and left incubate for an additional hour at 27°C. 7mL of 4% agarose at 60°C and 21 mL of warm 1.3x SF-900 was added to each well and incubated in lock boxes with moist paper towels for 4 to 6 days at 27°C until plaques were formed.

Confirmation of protein production

Antibodies were unavailable for CG17560 protein detection. To infer recombinant protein production, time course infection and RT-PCR was used to detect recombinant gene transcription. RNA was isolated from infected SF9 cells using the RNeasy Plant Mini Kit (Qiagen) with manufacturer's instructions. Prior to kit use, cells were centrifuged at 3000 rpm in the Beckman GS-6R centrifuge for 5 min at 4°C. A time course infection of CG17560 into SF9 cells was conducted by collecting RNA daily over five days for a total of five time course samples to view CG17560 RNA concentrations by qRT-PCR . The cDNA was synthesized in a reaction containing 12 µl template RNA from each sample, 250 pM random primers, 10 mM DNTPs, 0.1 mM DTT, 4 µl First strand buffer (Invitrogen) and 0.5 µl Superscript III Reverse Transcriptase (Invitrogen), for a final reaction volume of 19.5 µl, with the following cycling conditions; RNA, dNTPs and Random primers were incubated at 65°C for 5 min then 4°C for 1 min, at this point 1st Strand Buffer, DTT and Superscript II were added. Reaction was incubated at 25°C for 5 min, then 50°C for 1 h, then 70°C for 15 min with a final hold at 4°C. Finally, cDNA was then amplified with the appropriate gene specific primers (Table 1). To check

protein presence in both 17560 and CPR-infected cells, an SDS-PAGE gel stained with Coomassie blue was run with dilution factors of none, five, ten, and fifty.

Functional assays

Harvested protein was used to perform enzymatic functional assays as outlined in Table 2 and summarized below.

To produce recombinant FAR 17560 and negative control CPR in Sf9 cells, 50 ml liquid cultures were seeded at a concentration of $0.8x10^6$ cells/ml with 10 % fetal bovine serum (FBS) in SF-900 media with addition of appropriate inoculum volumes for infection by 17560 and CPR, respectively. The cultures were incubated at 27°C with shaking at approximately 1,300 rpm on orbital cell culture shakers for 72 h, with FFA substrates added after 48 h, and then centrifuged at 3000 rpm in the Beckman GS-6R centrifuge for 10 min at 4°C. The supernatant was disposed and the pellet was washed twice by resuspending it in 5 ml of 100 mM Tris-HCl pH 7.0 and centrifuging afterwards. The washed pellet was resuspended in cell lysis buffer (CLB), 10 ml 100 mM Tris-HCl pH 7.0, 100 μM DTT, 0.5 mM PMSF and 10 μl of protease inhibitor cocktail. Three ml aliquots of the CLB were used to resuspend the pelleted cells. Suspensions were sonicated with 15 one second bursts repeated 3 times before continuing onto functional assays. Recombinant housefly cytochrome P450 reductase CPR served as a negative control during functional assays.

Long chain free fatty acids (28:0, 26:0, 24:0, and 18:0) were purchased from Sigma Aldrich (St. Louis, Mo.). In the functional assays, free fatty acid substrates were suspended in 1ml 0.6% w/v CHAPS in SF9 media and heated until dissolved. The

resulting solution was cooled to room temperature before direct addition to 17560- and CPR-infected SF9 cells for overnight incubation. For each functional assay involving long chain acyl-CoA molecules, substrate and cell lysate supernatant [suspended in 100mM Tris HCl pH 7.0, 100 µM DTT, 0.5 mM PMSF and 10 µl of protease inhibitor cocktail] were combined to a total reaction volume of 600-1000 µl. All assay samples were extracted twice with hexane:ether (50/50, by volume) into glass vials and were dried completely with N2 gas and resuspended in pure hexane for GC analysis.

Gas Chromatography Analysis

Products from 17560 and CPR enzyme functional assays were analyzed using a DB-5 column (Agilent). Analysis for assays using Free Fatty Acids as a substrate had the following settings: injector 150°C, FID- 300°C. 160°C for 0.2 min, 160°C to 265°C at a rate of 15°C/min, 265°C to 295°C at a rate of 5°C/min and held for 5 min at 295°C.

Tables and Figures

Primer Name	Sequence
pENTR F2	GCGTTTCTACAAACTCTTCC
pENTR R2	GCAATGTAACATCAGAGATT
BacF1	AAATGATAACCATCTCGC
BacR2	GTTAGGGATAGGCTTCCC
CG17560 Infus F	actggatccggtaccATGGATAGCGAGATACAAG
CG17560 Infus R	atctcgagtgcggccCTAAACTACAAGTCTGGCC
CG17560 F2	CCG CCG AGG TGC AGA TTG TA
CG17560 F3	cct gcc act gtc cat ctt cc

Table 1. Oligonucleotide primers. Gene specific primers for CG17560 and other vector primers used. Red nucleotides indicate the pENTR portion of Gibson assembly primers.

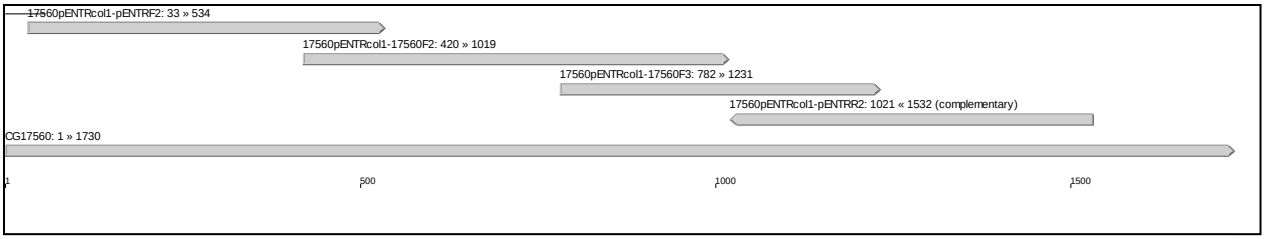

Figure 2. Full length Contig Sequence of CG17560 in pENTR.

ATGGATAGCGAGATACAAGGATTCTTTAAGAACAAGACCGTGTTCCTTACGGGTGGCACCGGATTTCTGGGAAAAG
TGATCATTGAGAAACTACTGAGGACCACGGAAGTCAATCGGATTTACTCCTTAATTAGACCTAAGCGGGGAGTGCCT
ATCGAGGACCGAATCACAACCTGGGCAAAGGATCCGGTGTTTGAGGTGCTCCTAAGGACGAAGCCAGATGCCTTGC
AGCGAGTTTGTCCCATCGCGGGCGACTGCTTGGATCCGGATCTGGGTATAAGCCAATCGGATCAAAGGATCCTGAC
CGCCGAGGTGCAGATTGTAATCCATGGAGCTGCAACCGTGCGATTCGACGAGGCTTTACACATCTCCTTGGCCATCA
ATGTACGAGCCACTCGTCTGATGCTTCAACTGGCCAAGCAGATGACCCAGCTGGTGTCCTTCGTTCACGTATCCACTG
CCTACTCCAACTGCGTGGTGCATGACATCGCAGAGCGATTCTATCCGGAGCACTTGAACTGCAGTTCGGATAAGATC
CTAGCCGTGGGTGAACTGGTAAGCAATAAATTACTGGACGCGATGGAACCTAGCCTTGTTGGCTCCTTCCCCAACAC
GTACACCTACACCAAAGCCCTGGCCGAGGATGTGATCCTGCGGGAGGCGGGCAACCTGCCACTGTCCATCTTCCGA
CCGGCGATCATAATGTCCACTTACAAGGAGCCCCTGGTTGGATGGGTGGACAACCTATTCGGACCACTGGCCCTCTG
CTTCGGCGGAGCTCGCGGTATAATGCGGGTCACCACGGTGGACCCTTCAGCCAAGATCAGCCTAGTTCCCGCTGACT
ACTGCGTAAATGTGGCATTGGCTTGCGCTTGGAGAACGGCGGAGATATCCGTACAGAATGGAAAGGTCACGACGCC
CCCCATTTACGCGTTTGCACCTAGCGAGAATAACCTGATGAGCTACGGAAACTTTATCAAATCATCTATTATCTATCG
GGACATCATACCGCTAACCAAGATGCTGTGGTATCCCTTCGTCCTGTGCATCTCCACCACATCCCTATTTCCGCTGGC
CGCCTTCTTCTTACACACCCTTCCCGGCTACTTCTTCGATCTGCTGCTGCGCCTCAAGGGCCGGAAGCCCATACTCGT
GGATCTGTATCGGAAGATTCACAAGAACATCGCCGTCCTGGGTCCGTTCTCCAGTACCACCTGGAACTTTGACATGA
CCAACACAATGGAGCTAAGGGAAGCGATGTCCAAGCAGGATCGCAACCTTTACGACTTCGACATGGCCCAGTTGGA
TTGGAATGACTATTTCAAGGCCGCCATGTATGGAATGCGTCTCTATATTGGCAAGGAGAAACCCACCGCGGAGTCCA
TTGCCAAGGGTCTGAAGCTGAGGATGCGCCTGAAGGTCCTTCACTATGCATTCGCATCCTCGCTGGTTGCTTTAGCT
GGTTACATCCTGTATTCTCTGGCCAGACTTGTAGTTTAG

Translation:
MDSEIQGFFKNKTVFLTGGTGFLGKVIIEKLLRTTEVNRIYSLIRPKRGVPIEDRITTWAKDPVFEVLLRTKPDALQRVCPIAG
DCLDPDLGISQSDQRILTAEVQIVIHGAATVRFDEALHISLAINVRATRLMLQLAKQMTQLVSFVHVSTAYSNCVVHDIAE
RFYPEHLNCSSDKILAVGELVSNKLLDAMEPSLVGSFPNTYTYTKALAEDVILREAGNLPLSIFRPAIIMSTYKEPLVGWVDN
LFGPLALCFGGARGIMRVTTVDPSAKISLVPADYCVNVALACAWRTAEISVQNGKVTTPPIYAFAPSENNLMSYGNFIKSSI
IYRDIIPLTKMLWYPFVLCISTTSLFPLAAFFLHTLPGYFFDLLLRLKGRKPILVDLYRKIHKNIAVLGPFSSTTWNFDMTNTM
ELREAMSKQDRNLYDFDMAQLDWNDYFKAAMYGMRLYIGKEKPTAESIAKGLKLRMRLKVLHYAFASSLVALAGYILYSL
ARLVV.

Figure 2. Full length nucleotide sequence and translation of CG17560 in pENTR.

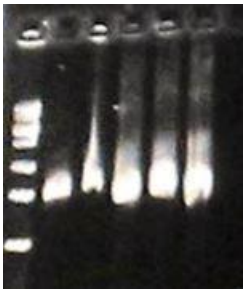

Figure 3. Time course results for CG17560 cDNA infection. The PCR reaction shows the increase in cDNA concentration as follows: 0 h, 24 h, 48 h, 72 h, and 96 h. All show increasing intensity of bands, indicating RNA production in the transfected cells and thus probable protein expression.

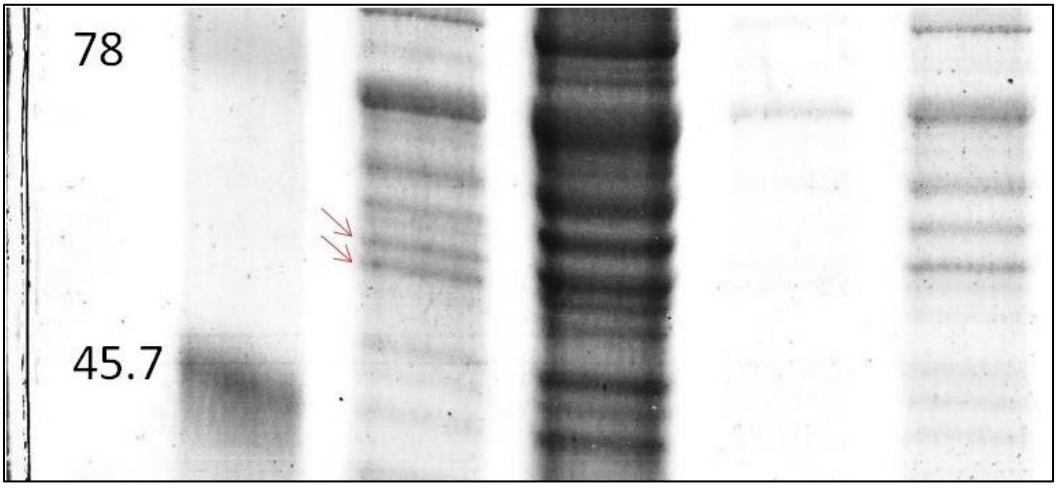

Figure 4. SDS-PAGE gel of FAR 17560 vs. CPR. Arrows point to protein bands present only in 17560. FAR 17560 is predicted to be 56 kDa. The lanes are from left to right: Ladder, FAR 17560 (neat, no dilution), CPR (neat, no dilution), FAR 17560 (1:5 dilution), CPR (1:5 dilution).

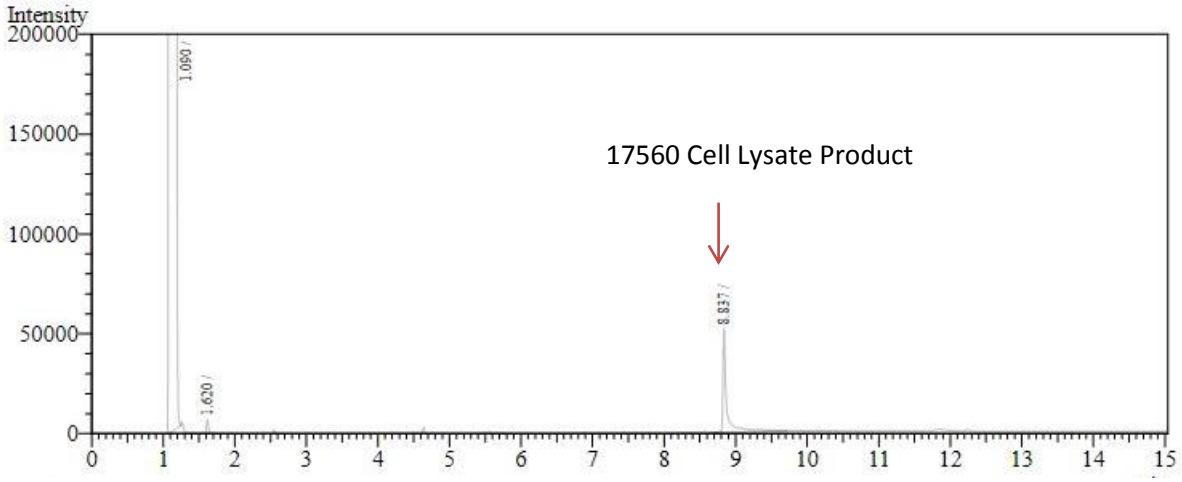

Figure 5. GC results for Cell Lysate of Sf9 Cells treated with 17560 viral stock and FFA Cocktail. The initial results for 17560 shows a characteristic peak at 8.837 min and was submitted to the Nevada Proteomics Center for GC/MS for analysis.

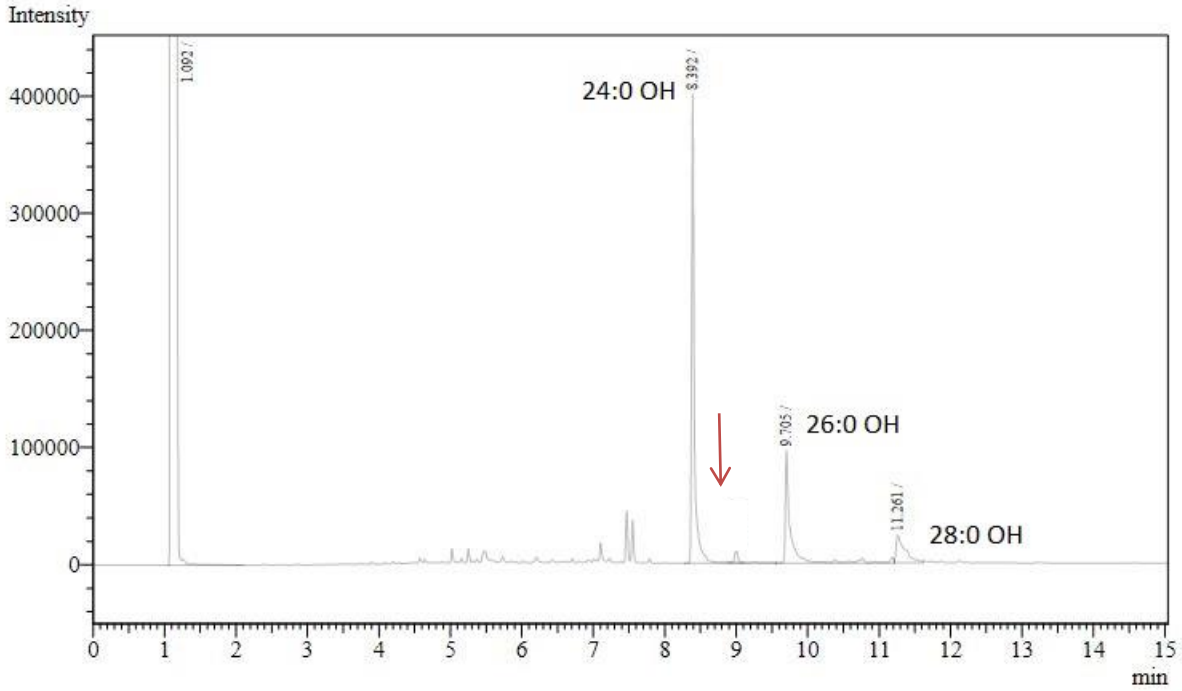

Figure 6. GC results for FFA OH Cocktail Standards. The product of 17560 in figure 5 eluted at 8.837 (red arrow), which lies between the 24:0 and 26:0 OH standard peaks and indicated a possible aldehyde of 26:0.

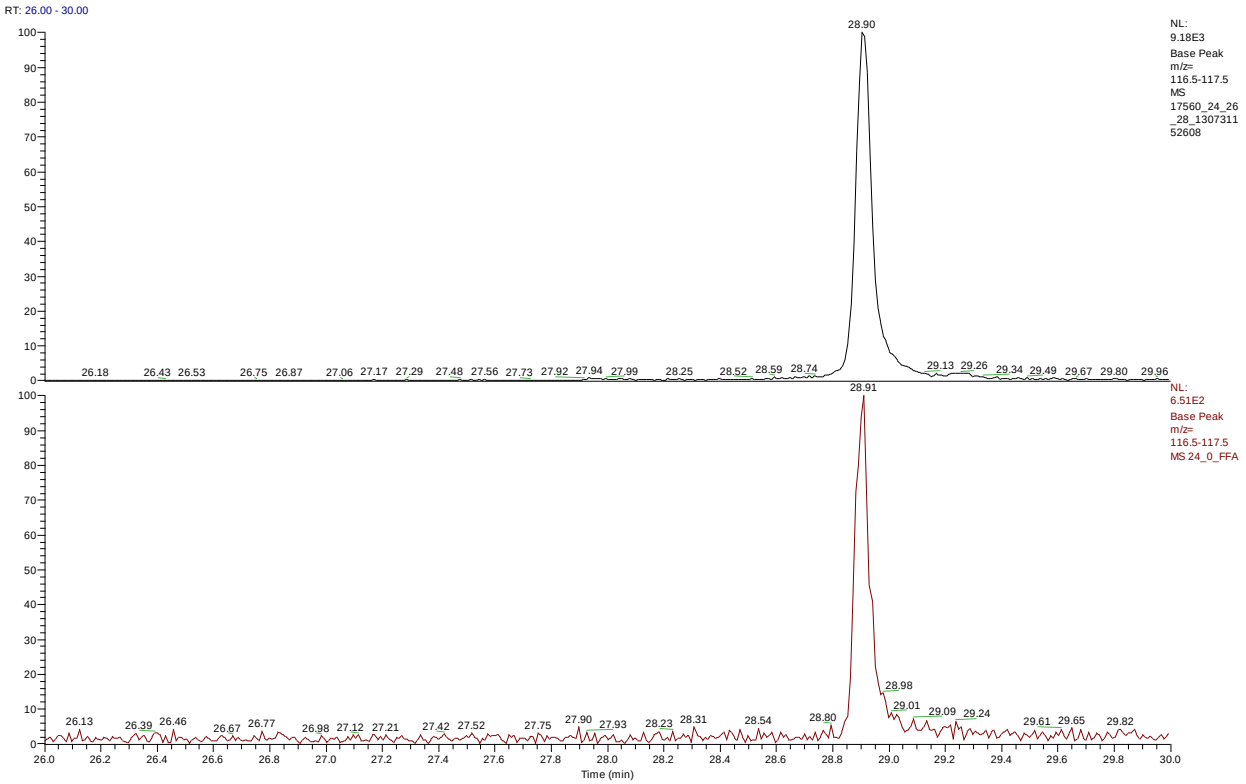

Figure 7. Gas Chromatography comparison Cell Lysate of Sf9 Cells treated with 17560 viral stock and 24:0 FFA. The characteristic peak shown at 28.90 min for 17560 (top) strongly suggests the presence of 24:0 FFA, which is shown as a standard peak at 28.91 min (bottom).

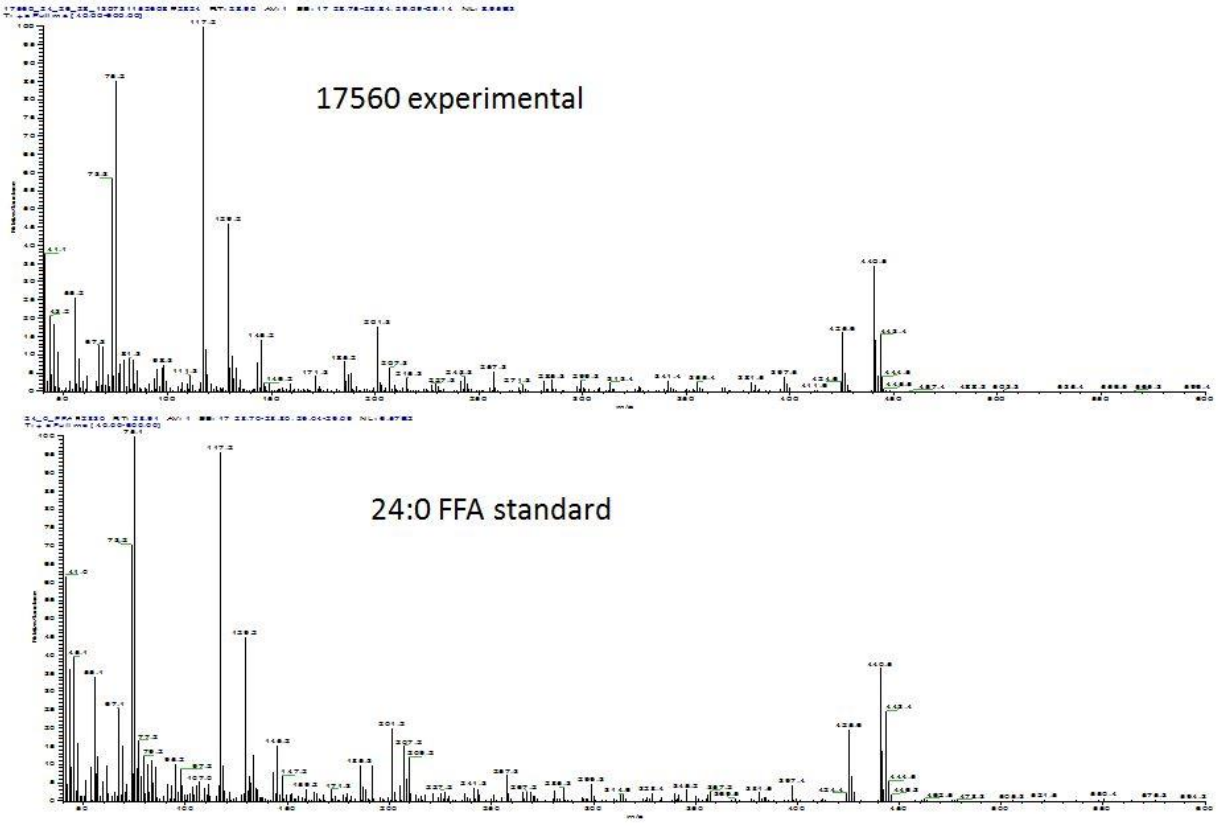

Figure 8. Mass Spectra comparison between Cell Lysate of Sf900 treated with 17560 viral stock and FFA Cocktail vs 24:0 FFA. Mass spectra data is shown with results from 17560 (top) and 24:0 FFA (bottom). There are three extremely characteristic peaks that are present in both that correlate to the presence of unaltered 24:0 FFA in the 17560.

Assay	Date	Substrate	Observed FFA	Notes
Replicate 1	7/19/2013	0.5mM FFA Cocktail (24:0, 26:0, 28:0)	24:0 FFA	No CPR Control
Replicate 2	7/23/2013	0.5mM FFA Cocktail (24:0, 26:0, 28:0)	none	
Replicate 3	8/27/2013	0.5mM FFA Cocktail (24:0, 26:0, 28:0)	24:0 FFA	No presence of 24:0 FFA in CPR
Dropdose 1	10/1/2013	0.4 mM (24:0 FFA)	24:0 FFA	Decreasing dilutions of viral infections used: 1:1, 1:2, 1:4, 1:8, and 1:16
Dropdose 2	10/15/2013	0.5 mM (24:0 FFA)	24:0 FFA	Increasing dilutions of viral infections used: 1x, 2x, 4x, 8x, and 16x
Replicate 4	11/27/2013	0.5 mM (18:0 FFA)	none	
Replicate 5	12/16/2013	0.5 mM (18:0 FFA)	none	
SF9 Test	12/16/2013	0.5 mM (18:0 FFA)	none	SF9, not infected, given 18:0 FFA to verify that passive FFA absorption not occurring

Table 2. Summary of Results for 17560 Functional Assays.

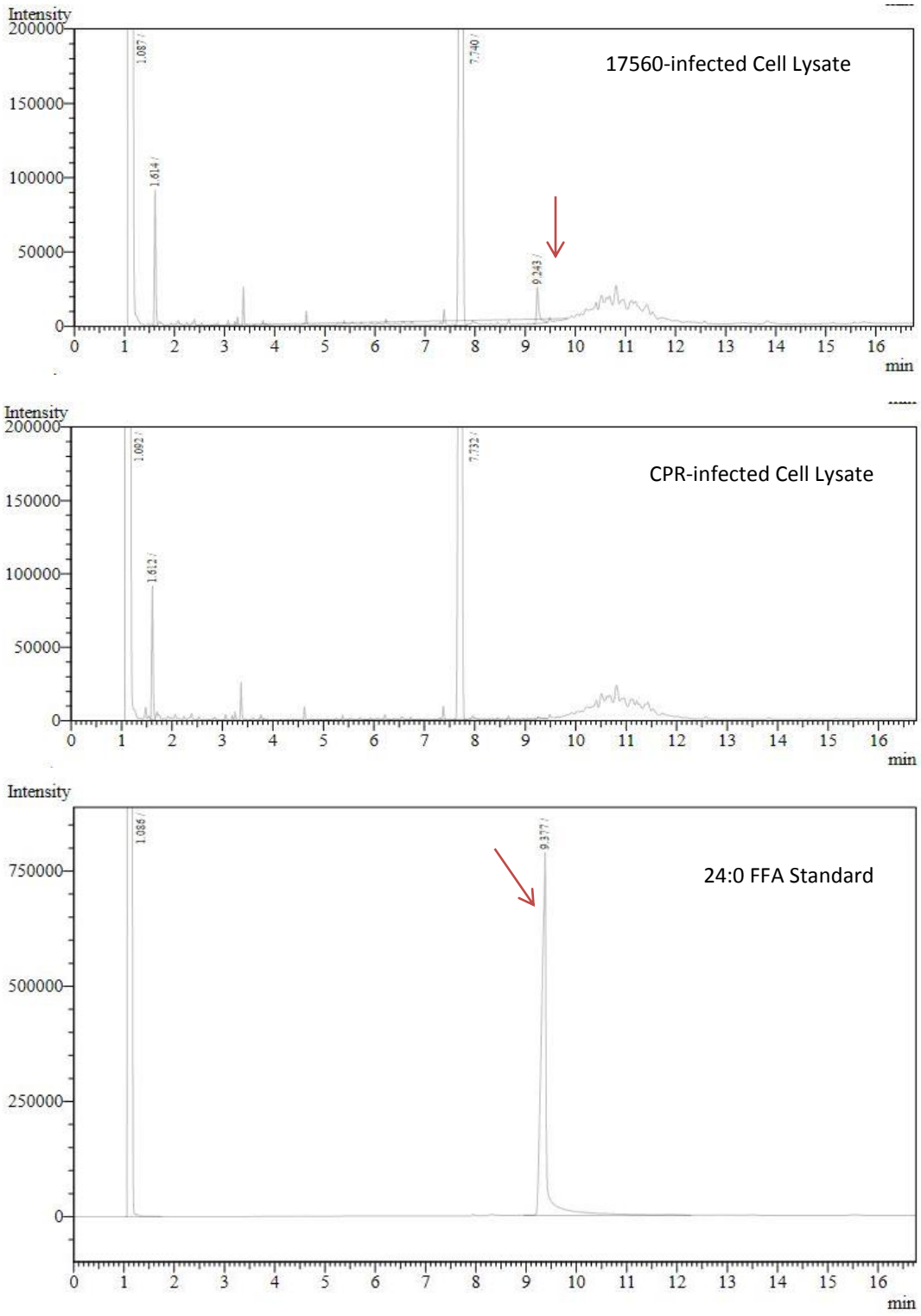

Figure 9. GC results of 17560, CPR, and 24:0 FFA Standard. 17560 (top) and CPR (middle) were treated with FFA cocktail. The peak at 9.243 min in 17560 most closely correlated to a 24:0 FFA peak when compared to the 24:0 FFA standard (bottom).

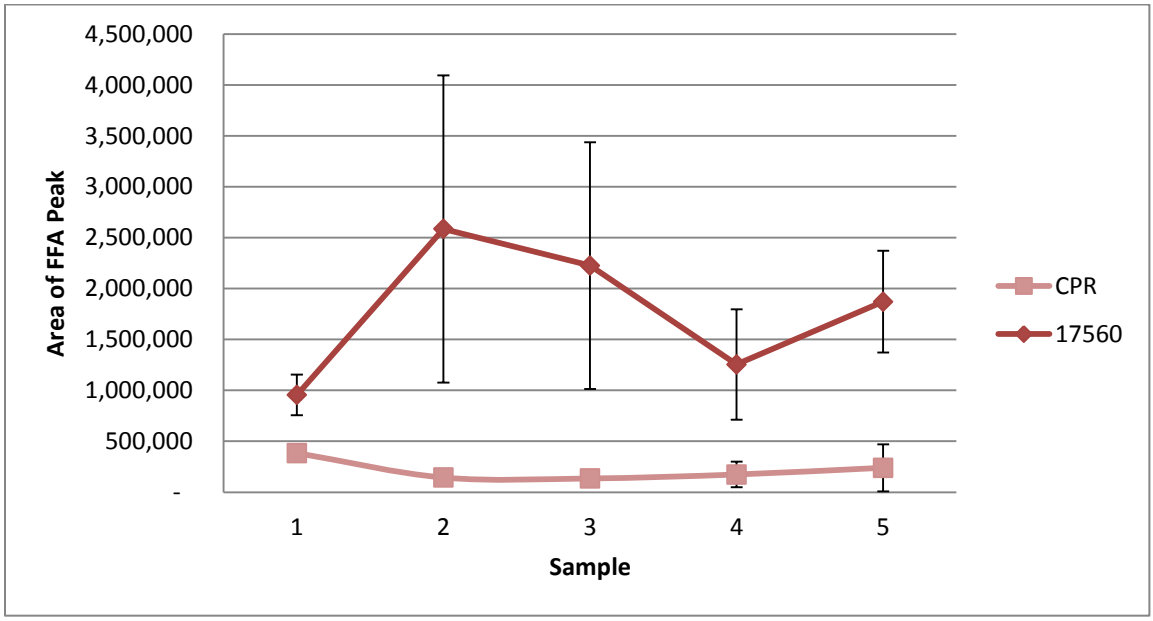

Figure 10. 17560 vs CPR dropdose 2 results for 2x increase in MOI per sample. Each sample of CPR and 17560 was treated with increased viral infection volumes to test for presence of 24:0 FFA in correlation controlled concentration of substrate.

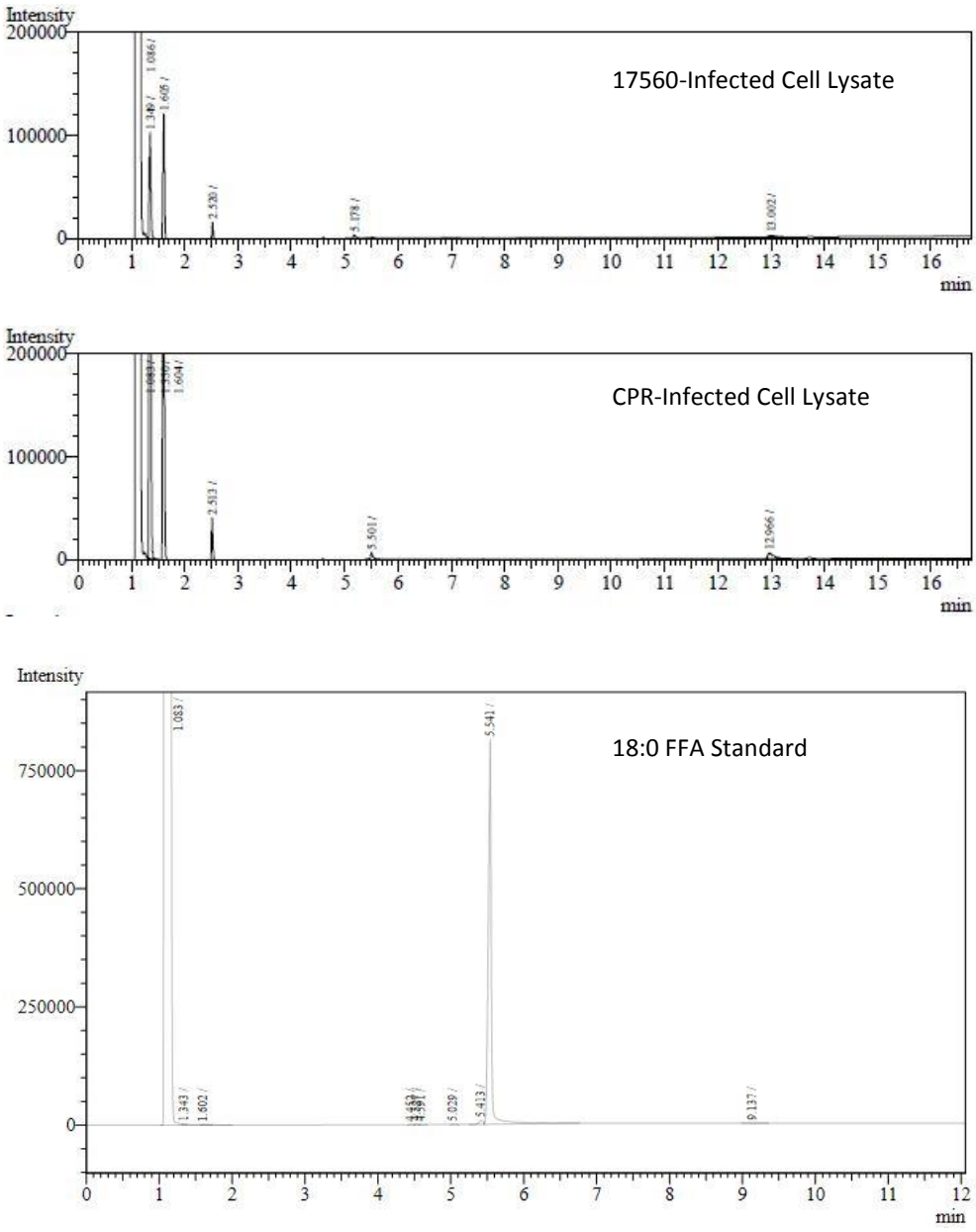

Figure 11. GC results of 17560, CPR, and 18:0 FFA. Both 17560 (top) and CPR (bottom) were treated with 18:0 FFA. Neither seemed to take up the 18:0 FFA, suggesting strong specificity of 17560 to 24:0.

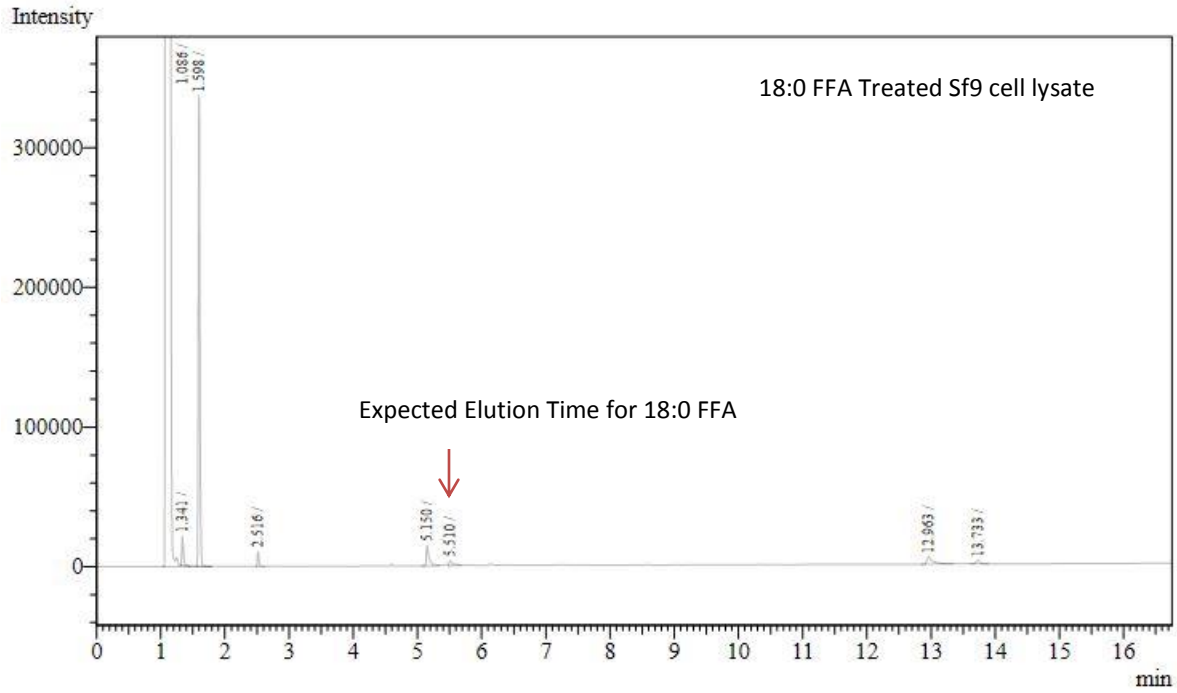

Figure 12. GC results of SF9 cells treated with 18:0 FFA. In order to verify that passive absorption of shorter length free fatty acids was not occurring, SF9 was treated with 18:0 FFA without any infection. There were no significant peaks to suggest absorption.

Results

Sequencing results of the entire CG17560 insert in pENTR showed no mutations, verifying the integrity of the insert by use of corresponding specific and vector primers (Table 1) and adequate overlap within the pENTR vector prior to transfer to baculovirus stock (Figure 1-2). Similarly, sequencing of the P3 viral stock was also confirmed to contain the CG17560 sequence prior to SF9 cell infection.

The plaque assay indicated that the titer of 17560 P3 was 2.3×10^8 pfu (plaque forming units). This titre is adequate for protein production, so the P3 stock was used to infect Sf9 cells for functional assays. CPR had a pfu of 6.5×10^7.

Recombinant 17560 mRNA levels in infected Sf9 cells were assayed RT-PCR. There was a qualitatively increasing intensity of the bands correlating to 17560 in the time course experiment, suggesting an increase in 17560 mRNA over time (Figure 3). Thus, it is inferred that a corresponding increased 17560 protein production in the SF9 cells over time was also occurring. The SDS-PAGE gel comparison between 17560 and CPR showed two bands in present in the cell lysate of Sf9 treated with baculoviral vector 17560 that were not present in the cell lysate of cells treated with the negative baculoviral vector control, CPR (Figure 4). These bands fell 51.4 kDa and 52.5 kDa with the predicted molecular mass of FAR 17560 being at 56 kDa.

Initial replicate experiments of 17560-infected SF9 cells treated with 24:0, 26:0, and 28:0 FFA cocktail showed a single GC peak corresponding to the product present in the cell lysate (Table 2, Figure 6). GC/MS analyses of the same sample revealed the experimental peak had a retention time and mass spectrum essentially identical to that of

untreated 24:0 FFA standard (Figure 7-8). This peak was not present in the CPR negative control.

A dose-dependence experiment of decreasing viral infections showed an overall decrease in 24:0 FFA detection with lower 17560-baculoviral doses whereas increasing viral infection led to presence of 24:0 FFA in the cell lysate (Table 2, Figure 10). Negative control dose-dependence experiments using CPR-baculoviral vector showed little to no detectable 24:0 FFA. To test the substrate specificity of the 17560, 24:0 FFA was replaced with 18:0 FFA (Figure 11). Both replicates that included 17560-infected cells and negative control CPR-infected cells showed no consistent or significant uptake of 18:0 FFA. The presence of a possible 18:0 FFA peak in CPR-producing (negative control) cells led to verification by treating SF9 cells with 18:0 FFA to observe if passive absorption was taking place (Figure 12). No significant peaks were seen.

Discussion

SDS-PAGE results showed a double band near 56 kDa correlating to FAR 17560 protein in Sf9 cells and are not present in the CPR-producing (negative control) cells, but due to the lack of an anti-17560 antibody it could not be confirmed if both were the same protein.

Sf9 cells producing recombinant 17560 retained 24:0 FFA, whereas other FFAs supplied to the media disappeared. The disappearance is presumably due to their conversion to fatty acyl-CoAs. Overall, there is some evidence that 17560 specifically binds 24:0 FFA. FFAs are normally converted to fatty acyl-CoAs before further metabolization, indicating that the disappearance of 26:0 and 28:0 FFAs were expected. However the persistence of 24:0 FFA in only 17560-infected Sf9 cells suggests specificity for this particular chain length. The data suggests that FAR 17560 is specific to 24:0 FFA and may be either binding and be unable to acetylate the FFA due to a missing conjugate protein or due to incompatible functional groups on the 24:0 FFA.

The initial GC results in figure 6 was suspected to be either 24:0 or 26:0 aldehyde due to its elution time between the 24:0 and 26:0 OH standard peaks. Further analysis revealed it was instead unmodified 24:0 FFA that was bound by FAR 17560 as seen in Figure 8. Further analysis of additional cell lysate functional assays revealed that the presence of 24:0 was specific for 17560-infected Sf9 cells and was not present in CPR-infected cells, indicating that 24:0 FFA uptake was specific to FAR 17560.

Given the apparent specificity of 17560 for 24:0 CoA binding, we expected a possible dose-dependent relationship. We tested this hypothesis by adding 24:0 FFA substrate to cells infected with different MOIs of 17560-baculovirus (and, by inference,

varying amounts of 17560 protein). GC analyses of these experiments confirmed that 24:0 FFA retention was specific for 17560-producing Sf9 cells. However despite predicting that increase of FAR 17560 and constant concentrations of 24:0 FFA would lead to complete uptake of all available 24:0 FFA given to the cells, it is shown that there is no direct correlation between increasing 17560 viral concentrations and concentration of 24:0 FFA (Figure 10). This is likely due to variable efficiency of the FAR 17560 production using the whole cell method. We note that 17560 protein concentrations were not measured as an antibody for the enzyme is not available. It is also possible that 17560 and 24:0 FFA concentrations were not within a range that would produce a reliable dose-response curve. Thus, the stoichiometry of binding has yet to be established.

It is unknown why the FFA is not modified by the enzyme, but it has been suggested that 17560 may work in conjunction with another protein not present in the SF9 cell. Following this, 17560 may act as a part of a series in a "metabolon" that works much like an assembly line to modify the products immediately as they are produced. Another explanation includes the specificity of 17560 in that it binds strongly to 24:0 FFA and prevents its conjugation to CoA, halting further metabolism by the cell, suggesting chain length specificity for the enzyme as well as that the 24:0 FFA may not have the appropriate functional groups to be acetified by 17560.

There were no significant peaks related to the treatment of 17560-infected cells with 18:0 FFA and the presence of small peaks in similar regions where 18:0 FFA would appear suggested either slight contamination from the functional assay or from the GC column itself.

The whole cell method has been somewhat successful in producing higher rates of working target proteins for functional assays compared to isolating the protein itself and observing it under highly controlled states, but poses a higher risk in unknown variables acting on the mechanism which may explain the presence of small peaks in GC results of SF9 cells fed with 18:0 FFA.

For future works, further testing of 17560 with 24:0 FFA using the isolated cell/complete functional assay should be done to remove all external factors that the whole cell method introduces. Anti-17560 antibodies should also be obtained to identify the two bands present in the SDS-PAGE analysis, to monitor protein production and also help with binding assays for competition of FFA binding sites. Radiolabeled substrates can also be useful in tracking the FFA within the 17560 pathway. Overall, additional research to determine the behavior behind 17560 can reveal further information about the mechanism behind the largely unknown FARs family and hydrocarbon biosynthesis.

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