Purification and Characterization of an Algal Dimethylsulfoniohydroxybutyrate Oxidase

By

Andrew P. Rideout

A thesis submitted to the

Department of Chemistry and Biochemistry

Mount Allison University

in partial fulfillment of the requirements for the

Bachelor of Science degree with Honours in Biochemistry

April 2023

TABLE OF CONTENTS

ABSRACT……………………………………………………………………………...…4

ACKNOWLEDGEMENTS……………… …………………………………………...5

LIST OF FIGURES………………………………………………………………….....6

ABBREVIATIONS……………………………………………………………….....7

INTRODUCTION

Biological Role of DMSP……………………………………………....……..9

DMSP in the Global Sulfur Cycle……………………………………………….11

DMSP Biosynthesis…………………………………………………….……..13

DMSHB Oxidase……………………………………………………………...…16

Class II Aldolases………………………………………………………………..17

Coupled Enzyme Assay……………………………………………………….…18

Research objectives………………………………………………… ………….…..19

MATERIALS AND METHODS

Chemicals and Reagents ……………...……………………………………………20

Protein Quantification……………………………………………………………20

Plasmids……………..…………………………………………………………21

Transformation and Cultures ……………………………………… ……………21

Protein Extraction……..……………………………………………… …………24

Plasmid Cloning……...………………………………………………… …………24

Protein Purification …...……………………………………………… …………25

Thrombin Cleavage Trials….………………………………………… …………26

Immunoblotting ………………………………………………………………… …28

GST-tagged protein Clean-up ……………………………………………………30

Coupled Enzyme Assay………..…………………………………………………32

RESULTS

Expression of Recombinant Proteins……………………………………………..35

Protein Purification…………………………………………………...……………36

Thrombin Cleavage Trials……………………………………………………………..40

Immunoblot of GST-tagged Recombinant Proteins……………………………...……42

GST Clean-up…………………………………………………………………...…..45

Work-up of DMOX Activity Assay……………………………………………..….48

DMSHB Oxidase Activity Assay……………………………………………..….49

DISCUSSION……………………………………………………………………………49

FUTURE DIRECTIONS……………………………………………………………….. .52

REFERENCES………………………………………………………………………. .53

ABSTRACT

Dimethylsulfoniopropionate (DMSP) is an organosulfur compound produced in large quantities in marine algae and is abundant in the ocean. DMSP is synthesized as a secondary metabolite in response to stress as an osmoprotectant, assisting in adaptations to increased salinity, high temperatures and freezing. Directly released DMSP is broken down to dimethylsulfide (DMS) by marine bacteria and by algae themselves. DMS is involved in the global sulfur cycle and implications in climate regulation. DMSP is biosynthesized in through a proposed four-step, enzyme-mediated pathway that has the amino acid methionine as a precursor. The final step in the algal DMSP biosynthetic pathway is the oxidative decarboxylation of dimethylsulfoniohydroxybutyrate (DMSHB) to DMSP facilitated by a DMSHB oxidase. This is the most poorly characterized enzyme in the DMSP biosynthesis pathway, as the only information is that molecular oxygen is incorporated into DMSP's carboxyl functional group.

This research built upon previous work on plasmid design and protein expression for recombinant forms of *Ulva mutabilis* DMSHB oxidase. Recombinant proteins were expressed and extracted from bacterial hosts in working quantities for future purification and enzymatic assays. Purification was carried out on 4 affinity-tagged recombinant proteins, two Glutathione *S*-transferase tagged and two hexahistadine-tagged, with associated protein tag affinity resins. GST- tags were successfully cleaved from recombinant DMSHB oxidase with thrombin, but separation of the free recombinant protein was unsuccessful. All recombinant proteins were tested for DMSHB activity using a fluorometric coupled enzyme assay, in which activity was not successfully measured. This study has advanced our understanding of DMSHB oxidase, through the success of protein expression.

ACKNOWLEDGEMENTS

I would first like to thank my supervisor, Dr. Jeff Waller for being an amazing mentor and a well of knowledge with everything molecular microbiology and biochemistry. This opportunity has afforded me much experience that will contribute to my future success.

I would also like to thank Dr. Tyson MacCormack for acting as my second reader in addition to enlightening me with so many cool animal facts. Thank you to Shafkat Ahmed for providing guidance in the lab in addition to the companionship within the Waller Lab. Thank you to Ca Nguyen for purifying some of the proteins used in this experiment.

Thank you, Mount Allison Department of Chemistry and Biochemistry, for making my past four years wonderful. I would also like to thank all of the lab instructors I have had throughout my time at Mount Allison for showing me the basics of lab techniques and etiquette. To all of the friends I have made at Mount Allison, thank you for forcing me to have a life outside of academics.

Lastly, I would like to thank NSERC, NBIF and Mount Allison University for making this opportunity possible through funding equipment and providing lab space. I would also like to thank the Dr. L.A. Goodridge endowment for funding me so I could carry out research over the summer.

LIST OF FIGURES

Figure 1. Chemical Structure of dimethylsulfoniopropionate (DMSP).

Figure 2. Proposed model of cycling of DMSP/DMS between marine ecosystems and atmosphere.

Figure 3. DMSP biosynthetic pathway from higher plants *W. biflora* and *S. alterniflora*.

Figure 4. The current proposed DMSP Biosynthesis pathway in marine algae.

Figure 5. Enzymatic assay scheme for measuring DMOX activity by following NADH oxidation to NAD+ with absorbance at 340 nm (A340) or fluorescence (λex=340 nm, λem=415 nm).

Figure 6. Expression of Um36.102.1His6, Stag.T.Um36.102.1, and GST.l.DMOX in *E. coli* RIPL.

Figure 7. Expression of Um145.His6.IF and Um36.102.1 in *E. coli* RIPL.

Figure 8. SDS-PAGE to monitor purification of GST.l.DMOXv2/v3 using a GSH-sefinose gravity column.

Figure 9. SDS-PAGE of GSH-sefinose column-purified GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins from the first extract of round 3 of culturing.

Figure 10. SDS-PAGE of GSH-sefinose column-purified GST.l.DMOXv2 and GST.l.DMOXv3 from the second extract of culturing round 3

Figure 11. SDS-PAGE of Nuvia Ni^{2+}column purified Um145.His6.IF and Um36.102.1His6 recombinant proteins

Figure 12. Thrombin Cleavage trial 1 of GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins

Figure 13. Thrombin cleavage trial 2 of GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins

Figure 14. SDS-PAGE in preparation for immunoblotting to confirm protein identity of GST.l.DMOXv2/v3.

Figure 15. Immunoblot detection of GST tag in progressive purification and cleavage of GST.l.DMOXv2 and GST.l.DMOXv3.

Figure 16. Immunoblot detection of DMOX in progressive purification and cleavage of GST.l.DMOXv2 and GST.l.DMOXv3

Figure 17. Progressive thrombin cleavage and clean-up attempt 2 of GST.l.DMOXv2 and GST.l.DMOXv3 extracts

Figure 18. Progressive thrombin cleavage and clean-up attempt 2 of GST.l.DMOXv2 and GST.l.DMOXv3 extracts

Figure 20. Batch method for clean-up of GST.l.DMOXv2 and GST.l.DMOXv3 NBEs with residual proteins at similar sizes

ABBREVEIATIONS

BGG	Bovine γ-globulin
BSA	Bovine serum albumin
cDNA	Complementary DNA
CNN	Cloud-condensing nuclei
CV	Column volume
DCM	Dichloromethane
DMS	Dimethylsulfide
DMSHB	4-Dimethylsulfonio-2-hydroxybutyrate
DMSO	Dimethyl sulfoxide
DMSP	Dimethylsulfoniopropionate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GST	Glutathione *S*-transferase
His_6	Hexahistidine
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny broth
LN_2	Liquid nitrogen
MeSH	Methanethiol
Met	Methionine
Met AT	Methionine aminotransferase
D-MTHB	*D*-Methylthiohydroxybutyrate

MT	MTHB *S*-Methyltransferase
MTOB	Methylthio-2-oxo-butyrate
NADPH	Nicotinamide adenine dinucleotide phosphate
NBE	Non-binding eluate
NP	No polylinker
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
SOC	Super optimal broth with catabolite repression
SDS	Sodium dodecyl sulfate
SAM	*S*-adenosyl methionine
SMM	*S*-methyl methionine
TAE	TRIS-acetate-EDTA buffer
TBS-T	TRIS-buffered saline with Tween-20
TRIS	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
v/v	Volume / volume
w/v	Weight / volume

INTRODUCTION

Biological Role of DMSP

Life on Earth needs sulfur to carry out many of the necessary processes, one example being protein synthesis, In the environment, there is only a small amount of sulfur bound in biomass at one time, totaling around 1% of dry weight in organisms (1). Sulfur species serve multiple roles as amino acids (cysteine and methionine), as coenzymes (*e.g.* biotin and thiamine), commonly in the form of iron-sulfur clusters, and other biomolecules (1). Terrestrial organisms have decreased levels of access to sulfur, but it is in high quantities in marine environments (1). Inorganic sulfurs can be utilized by microorganisms through two processes, assimilation is the energy-dependent conversion of inorganic to organic sulfurs, whereas dissimilatory sulfur metabolism uses sulfurs as electron acceptors in oxidation-reduction reactions (1, 2). The high level of sulfur becomes important to other marine bacteria as it is commonly used as an electron carrier when conditions become hypoxic or anoxic (1, 3).

Assimilatory and dissimilatory responses in marine microorganisms contribute to the rapid and vital process of sulfur cycling. This initially takes place in the surface water with the uptake and assimilation of sulfates into trace polysaccharides, but primarily into cysteine and methionine in cyanobacteria and algae (1). Methionine can be further metabolized for additional cellular functionality to dimethylsulfoniopropionate (DMSP), a stable and soluble sulfur depository (1, 4, 5). DMSP is synthesized and stored in large quantities in dinoflagellates, prymnesiophytes and chrysophytes (intracellular concentration of 100-300 mM) and to a lesser extent in diatoms (≤50 mM) (1, 3). Since DMSP contains 5 carbons it is also a large source of carbon in marine environments, making up 3-10% of marine primary production of carbon and of the carbon requirements of surface water heterotrophic bacteria
(1).

DMSP is a tertiary sulfonium compound and is synthesized in high quantities in some genera of marine algae, in response to stress. DMSP is zwitterionic, meaning it has a both positive and negative charge within the same molecule at a given pH, as seen in figure 1 (6). Zwitterions have known uses and multiple types have been identified in living things, the most common of which is the group of molecules known as betaines (7). Betaines are amino acid derivatives characterized by their quaternary ammonium groups and carboxylic acid (7). Glycine betaine, proline betaine and β-alanine betaine are the most common, each being found in both plants and microorganisms (7). DMSP is analogous to these betaines, where instead it contains a tertiary sulfonium instead of quaternary ammonium, both DMSP and betaines have similar chemical and physiological properties (7). Betaines and DMSP are used as a primary osmoprotectants, and as such their production levels rise with increasing salinity. DMSP production can be advantageous over betaine production in when nitrogen is scarce since the production of betaines are nitrogen dependent (7).

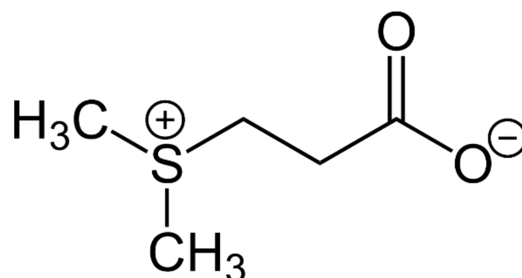

Figure 1. Chemical structure of dimethylsulfoniopropionate (DMSP). DMSP is zwitterionic as evidenced by the positively charged sulfonium and negatively charged carboxylic acid.

DMSP has more beneficial effects than the aforementioned. Increased levels of DMSP have been observed in response to freezing temperatures, thus acts as a cryoprotectant (8). Accumulating high levels DMSP and the products from its breakdown have also been shown to readily scavenge hydroxyl radicals and lower oxidative stress (9). This antioxidant activity can

be in response to UV-light and low levels of Fe or CO_2 , where DMSP and associated molecules neutralize reactive oxygen species (9).

DMSP in the Global Sulfur Cycle

Algae play a critical role in biogeochemical processes, with increased importance in cycling essential elements or life. There are 5 elements that need to be constantly cycled between environmental compartments, these elements are carbon, nitrogen, oxygen, phosphorus, and sulfur (CNOPS) (10). The focus in this study however will be on sulfur and the different forms it is found as in marine environments.

DMSP is released into the environment in a multitude of ways. Algae are considered primary producers and are subject to grazing by zooplankton, cellular lysis caused by viral infection, and senescence (11–13). Dissolved DMSP can be readily broken down into dimethylsulfide (DMS) in marine surface waters by bacteria and other algae, (Figure 2) (14). DMS is volatile and acts as a primary contributor to the sea-to-air flux of sulfur compounds, as it moves between these environmental compartments freely as driven by wind (11). The movement of DMS in the air mediates the movement of large amounts of biogenic sulfur from the ocean to terrestrial environments completing the cycle between the spheres that need sulfur to sustain life (14).

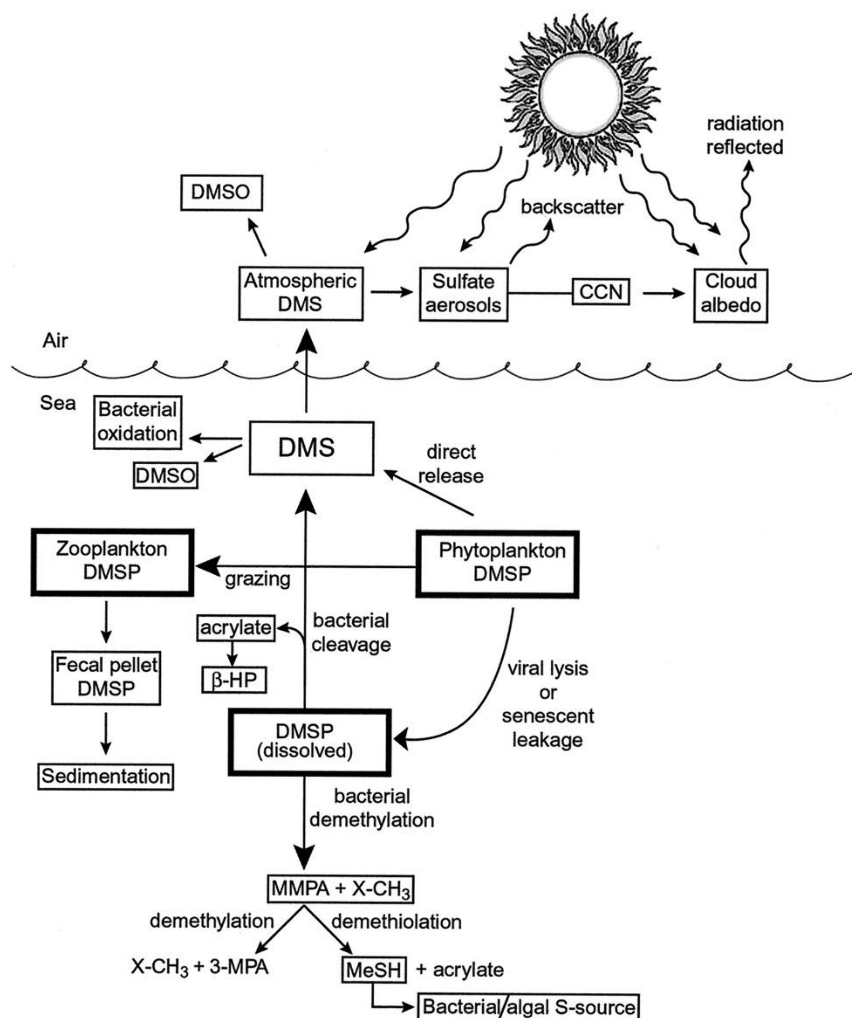

Figure 2. Proposed model of cycling of DMSP/DMS between marine ecosystems and atmosphere. Marine DMSP primarily becomes DMS that will enter the atmosphere, but there are alternative fates for DMSP illustrated here. DMSO, dimethyl sulfoxide; CCN, cloud-condensing nuclei; MMPA, 3-methiolpropionate; β-HP, β-hydroxypropionate; 3-MPA, 3-mercaptopropionate; MeSH, methanethiol and X-CH3, unidentified molecule with a terminal methyl group. Figure courtesy of Yoch (2002).

The CLAW hypothesis proposes that DMS is the major contributor to sulfur species in the atmosphere that contributes to cloud condensation nuclei (CCN) (11). This increase in CCN would lead to increased cloud albedo, which increases the radiation reflected away from earths surface which decreases temperature in a location (11). This creates a proposed negative feedback loop where increased water temperature and UV-radiation increases the production of DMSP to combat stress, these high levels of DMSP would increase the amount of DMS (11).

The DMS would lead to more cloud cover which lowers temperature and UV exposure, decreasing the need for DMSP. The importance of DMSP on cloud cover has been questioned in the years since, and as such its contributions have been more accurately determined, as human activity now dominates the global sulfur cycle (15).

DMSP Biosynthesis

Global biosynthesis of DMSP is believed to be dominated by marine varieties of micro- and macro-algae, with a lesser amount being released from a handful of halophytic plants. To this point, there have been three DMSP biosynthesis pathways that utilize methionine as a precursor, each of these pathways believed to have evolved separately. Plants have two, presumed divergent, pathways to produce DMSP, these pathways have been identified in *Wollastonia biflora* and *Spartina alterniflora* (Figure 3) (6). Both start with the methylation of methionine to *S*-methyl methionine. *W. biflora* then goes though transamination and decarboxylation to reach DMSP-aldehyde (16). In *S. alterniflora, S*-methyl methionine is decarboxylated first then undergoes transamination to DMSP-aldehyde (17). The DMSP-aldehyde produced by both is then oxidized to DMSP in the same manor (16).

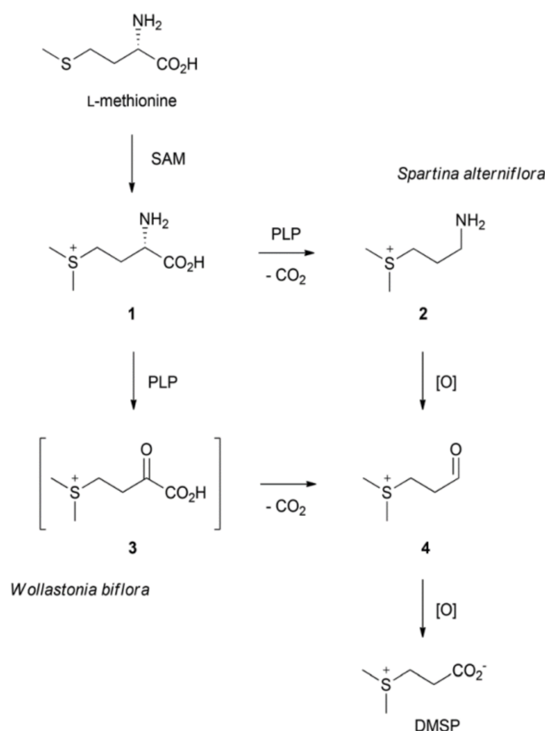

Figure 3. DMSP biosynthetic pathway from higher plants *W. biflora* and *S. alterniflora*. First and last reactions are conserved in each plant but differ in their intermediate reactions. Individual reactions are outside the scope of this investigation, used to show differences. Figure from Dickschat *et al.* 2015.

The DMSP biosynthesis pathway as identified in marine algae is carried out in 4 steps (figure 3). These steps are: 1) the reversible transamination of methionine to 4-methylthio-2-oxybutryate (MTOB) with α-ketoglutarate, 2) stereospecific reduction of MTOB to *D*-4-methylthio-2-hydroxybutyrate (MTHB) using NADPH, 3) which is methylated to 4-dimethylsulfonio-2-*D*-hydroxybutyrate (DMSHB) *via* a *S*-adenosyl methionine-dependent reaction, 4) oxidative decarboxylation of DMSHB to the final product of DMSP (4). These reactions are all enzyme catalyzed, but to date these enzymes have only been partially characterized and much more needs to be understood. The enzyme that initializes this pathway, methionine aminotransferase (Met AT), prefers α-ketoglutarate and methionine as substrates over other molecules and amino acids (5, 18). The second reaction is catalyzed with MTOB reductase which has been found to be stereospecific and highly preferential to NADPH over

NADH (5). The first two reactions have been observed in organisms outside of known DMSP producers and are believed to serve wider roles intracellularly. The third reaction is catalyzed by MTHB S-methyltransferase and is the first irreversible reaction suggesting that this is the committing step (4, 5, 19, 20). This reaction is also dependent on *S*-adenosyl methionine, early theories posed this enzyme as evolving early on to allow the use of DMSHB as relatively weak osmoprotectant (4). The last enzyme is still very understudied and little information is known on its function and is presently termed DMSHB oxidase (21, 22). The only characteristic of this enzyme to be elucidated to this point is that it incorporated molecular oxygen into the carboxyl group of DMSP while eliminating a carboxyl from the substrate (4).

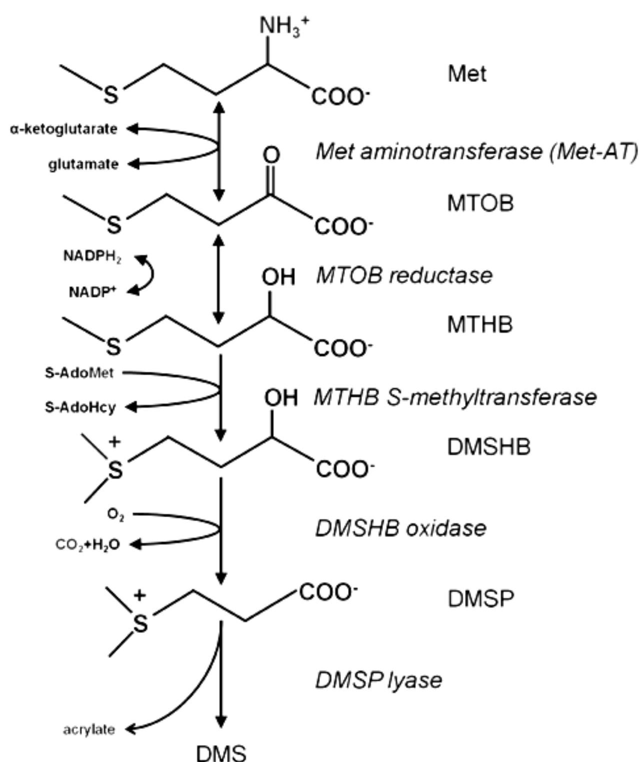

Figure 4. The current proposed DMSP Biosynthesis pathway in marine algae. Methionine (Met) undergoes a reversible transamination to 4-methylthio-2-oxybutyrate (MTOB), followed by a reduction to 4-methylthio-2-hydroxybutyrate (MTHB) that is also reversible. MTHB undergoes irreversible methylation to 4-dimethylsulfonio-2-hydroxybutyrate (DMSHB), which then undergoes an irreversible oxidative decarboxylation to Dimethylsulfoniopropionate (DMSP). DMSP lyase is an example of DMSP degradation in the environment.

DMSHB Oxidase

There is little known about the final reaction in algal DMSP biosynthesis, and the enzyme that catalyzes this reaction has yet to be categorized as a specific protein class, this is echoed in the lack of published information available. One of the first studies with the aim to elucidate more information on the marine algae biosynthesis pathway was done by Gage *et al.* (1997), which used radiolabelling to understand each enzyme. DMSHB was theorized to have oxygenase-mediated oxidative decarboxylation capabilities, with potential to be analogous to lactate oxidase (4). This is supported by having [$^{13}C_4$]-methionine undergo DMSP biosynthesis in *U. intestinalis* while in an $^{18}O_2$ atmosphere, where resulting DMSP had both ^{13}C and ^{18}O within the final DMSP molecules (4). This confirmed that DMSHB oxidase can decarboxylate DMSHB while utilizing molecular O_2.

However, this experiment was done *in vivo* and the oxidative decarboxylation reaction of DMSHB has never been done successfully *in vitro*. With this gap in knowledge researchers have only been able to speculate on characteristics and classification of this enzyme solely on its oxygenase activity. There are several enzymes that can do this, and it has been speculated that it is homologous to one of these other enzymes. A breakthrough in DMSP biosynthesis research has been made in the Waller lab (19, 21, 22) in which a gene within the *Ulva* genus thought to encode the last three enzymes in the proposed DMSP biosynthesis pathway has been identified. Further bioinformatic studies determined that this gene was highly conserved across DMSP-producing algae, and that this gene encodes a large, single polypeptide that has different domains: a reductase domain, 2 methyltransferase domains, and a class II aldolase domain. Methyltransferase activity has been confirmed with *D*-MTHB as the sole substrate as expected from previous studies, the reductase has yet to be fully characterized but is homologous to other

NADPH-dependent D-2-hydroxyacid dehydrogenases (5, 19, 20, 23). These results have led to the current suspicion that the class II aldolase is DMSHB oxidase.

Class II Aldolases

Aldolases are a superfamily of proteins that are divided into two subfamilies, that differ in catalytic mechanism, but serve the same function of cleaving C-C bonds (24). Class I aldolases catalyze these kinds of reactions by covalently linking lysine within the active site to the substrate of interest, allowing for the stereospecific deprotonation of the substrate (24). Class II aldolases are interesting because they require transition metals as cofactors, divalent metals more commonly, that facilitate substrate deprotonation through a proposed Lewis acid mechanism (25).

There are a few Class II aldolases that possess a suspected oxygenase activity, but very few of them have been fully characterized for their kinetics and this additional activity. An example of this is an enzyme responsible for 2 oxidative decarboxylation reactions in the production of clorobiocin (26). This enzyme is classified as a 4-hydroxy-3-prenylphenylpyruvate oxygenase/4-hydroxy-3-prenylbenzoate synthase but goes by the common name CloR after the gene. Experiments similar to those done by Gage *et al*. (1997) showed that ^{18}O was incorporated into intermediates of clorobiocin while synthesis was done in an $^{18}O_2$ environment (26). It showed similar activity and results to Gage *et al*. (1997), where external, molecular oxygen is incorporated into the substrate by these enzymes. The similarity between these two enzyme catalyzed reactions has led to our investigation of DMSHB oxidase as a class II aldolase.

Coupled Enzyme Assay

With the proposed similarities to CloR, and the proposed release of CO_2 along with DMSP has led to the assay implemented in this study. Production of CO_2 has been measured through fluorometrically by coupling it to the incorporation of HCO_3^- and phosphoenolpyruvate (PEP) to produce oxaloacetate (OAA) *via* the enzyme phosphoenolpyruvate carboxylase (PEPC), as seen in figure 5 (27). OAA can be reduced to malate through the malate dehydrogenase (MDH) enzymatic assay (27, 28). One caveat to this is OAA at high pH and in the presence of divalent metals, Mg^{2+} in this case, can be broken down into pyruvate (29), which can result in an underreporting of activity measured. To account for the loss of OAA, the production of pyruvate is coupled to the lactate dehydrogenase assay, oxidizing another NADH and taking pyruvate to lactate (29). The loss of NADH can be measured with absorbance at 340 nm or with fluorescence (λ_{ex}= 340 nm, λ_{em}= 460), which will be proportional to the production of DMSP.

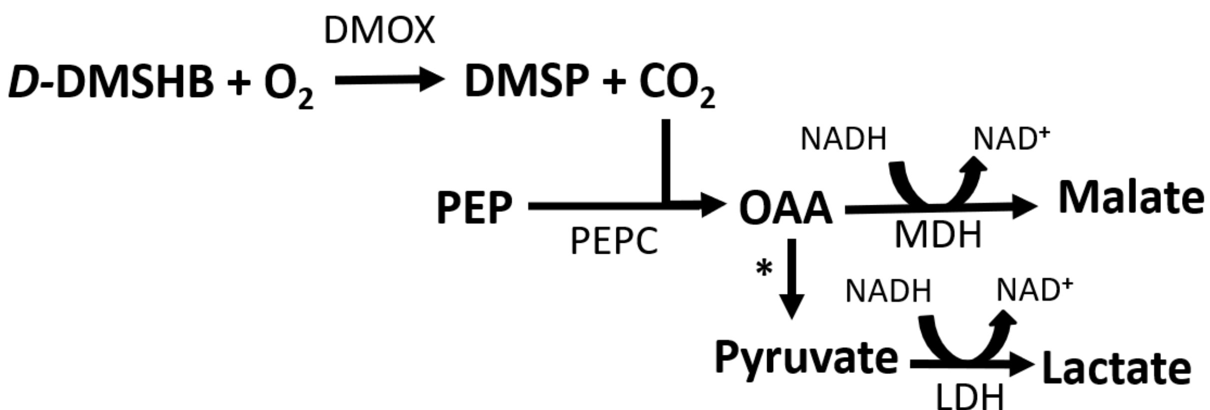

Figure 5. Enzymatic assay scheme for measuring DMOX activity by following NADH oxidation to NAD+ with absorbance at 340 nm (A340) or fluorescence (λex=340 nm, λem=415 nm). PEP, phosphoenolpyruvate, PEPC, PEP carboxylase, OAA, oxaloacetate, MDH, malate dehydrogenase, and LDH, lactate dehydrogenase.

Research objectives

The goal of this research project was to characterize the unstudied DMSHB oxidase enzyme involved in algal DMSP biosynthesis. This overarching goal required completing several smaller goals including: 1) Overexpression and purification of recombinant, tagged DMSHB oxidase, 2) Removal of protein tags while retaining DMSHB oxidase activity, 3) measure enzyme kinetics for the proposed DMSHB oxidase reaction. Each of these smaller goals will further the understanding of this pathway and global DMSP biosynthesis.

MATERIALS AND METHODS

Chemicals and Reagents

Each chemical and enzyme used was supplied from, unless stated to be otherwise, Sigma-Aldrich, Fisher Scientific, BioShop Canada, or BioRad. All the buffers and reagents were prepared using MilliQ ultrapure water and prepared at the temperature of use. Bacterial culturing work was done using aseptic technique in a laminar flow hood.

Protein quantification

Soluble proteins were analyzed through the colorimetric Coomassie protein assay as described by (30) using a standard curve of bovine gamma-globin (BGG, Thermo Scientific, 23212) from 0.0-0.4 mg/mL. Absorbance at 595 nm was measured with a Molecular Devices SpectraMax M3 microplate spectrophotometer and recorded with the SoftMax Pro program.

Insoluble proteins were solubilized in 4X Laemelli buffer solution (LBS; 200 mM TRIS-Cl (pH 6.8), 400 mM DTT, 8% w/v SDS, 0.4% w/v bromophenol blue, 40% v/v glycerol) and boiled for 5 minutes, then centrifuging at 10000 x *g*. Samples were blotted onto GE Healthcare chromatography paper (1 µL each) alongside BSA standards (0.1-10 mg/mL). Blotted chromatography paper was stained using 1% (w/v) Coomassie Brilliant Blue R250 (in 50% v/v methanol, 10% v/v acetic acid) for 1hr and destained overnight with 40% v/v methanol, 10% v/v acetic acid. Concentrations were estimated by comparing the color intensity of the dilutions to the standard curve.

Soluble and insoluble samples were prepared for gel analysis following the procedure outlined by (31), by diluting extracts to the required concentration in 1X LBS, and boiled the same as above. Sample preparations were tested for improved resolving with samples incubated with 2% (w/v) iodoacetamide and/or denatured at 75°C, at which 75°C was used for sample preparations as a precaution. Prepared samples were then run through either a 10% or 12% (v/v)

acrylamide tris(hydroxymethyl)aminomethane (TRIS)-glycine gels, with 1X SDS running buffer. Gels were resolved for 1 hr 20 min at 120V, stained with 1% (w/v) Coomassie Brilliant Blue R250 and destained with destain solution (45% methanol, 10% acetic acid).

Plasmids

Plasmids were supplied as dehydrated solids from Biobasic. Plasmids were rehydrated with sterile MilliQ and plasmid concentrations were determined using Nanodrop 1000 Spectrophotometer (Thermo Scientific) in a 1:1000 dilution. Samples were kept on ice until used in bacterial transformations and were stored long term at -20°C.

Transformation and Cultures

Electrotransformation of E. coli

Previously prepared plasmids were used in the electrotransformation of electrocompetent *Escherichia coli* (*E. coli*). Two different cell lines were used in these transformations, DH10β and BL21 CodonPlus (DE3) RIPL (hereafter RIPL), DH10β was used for amplification of cloned plasmids, RIPL was used for overexpression of protein. Electroporation was done with a MicroPulser electroporation system (BioRad) set to 2500V, with an anticipated time constant of >5.0 msec.

DH10β cells were transformed with four plasmids for the study of DMOX; Um36.102.1::pET28b, Um36.102.1His6::pET28b, and STag.T.Um36.102.1::pET29b. The solubilized plasmids (0.5 µL) were added into previously prepared bacteria aliquots (50µL), and electroporated as described above. RIPL cells were transformed with 6 plasmids; STag.T.Um36.102.1::pET29b, Um36.102.1His6::pET28b, Um36.102.1::pET28b, Um145.His6. IF::pET28b, GST.l.DMOXv2::pGEX2T-3, and GST.l.DMOXv3::pGEX2T-3. Plasmid solution (0.2 µL) was added to 50 µL of prepared electrocompetent RIPL cell aliquots, and electroporated

as above. Samples with a satisfactory time constant were then recovered into 500 µL of SOC media (Sigma-Aldrich, S1797). These tube cultures were incubated at 37°C for 45 minutes while shaking at 200 rpm.

LB (10 mg/mL tryptone, 5 mg/mL yeast extract, 10 mg/mL NaCl) solidified with 1% w/v agar was prepared following Roche guidelines (32) , media contained 50 µg/mL of antibiotic, kanamycin or ampicillin, and 1% w/v glucose. Each culture (25 µL) was applied to plates with the correct corresponding antibiotic. Plasmids and their corresponding antibiotic resistances are as follows; pET28b, pET29b to kanamycin, pGEX2T-3 to ampicillin. These plates were incubated at 37°C overnight.

Overexpression Cultures

Round 1:

Overnight cultures were prepared by inoculating 1-2 individual colonies from agar plates prepared previously, into 5 mL of LB-phosphate media (LB media buffered with 0.1 M sodium phosphate (pH 7.8), 50 µg/mL of either kanamycin or ampicillin, 1% w/v glucose). RIPL cell cultures expressing 4 plasmids were used in the first round of culturing; STag.T.Um36.102.1::pET29b, GST.l.DMOXv2::pGEX2T-3, GST.l.DMOXv3::pGEX2T-3, and Um36.102.1His6::pET28b. Cultures were incubated overnight at 37°C and 200 rpm. The following day, cell growth was measured using the absorbance at 600 nm (A600) of three dilutions 1:5, 1:10 and 1:20. The average was used to determine the inoculation volume needed to achieve an OD of 0.05 for day cultures. In each case the OD was below the optimal starting density, inoculation proceeded with the whole remaining culture.

Day cultures were made by inoculating sterile 400 mL of LB-phosphate media in 2 L baffled flasks, with the whole volume of each corresponding overnight culture. Ampicillin or

kanamycin was added to achieve 50 µg/mL in the final volume, the media also had 1x trace metals (20µM $CaCl_2$, 10µM $MnCl_2$, 10µM $ZnSO_4$, 2µM $CoCl_2$, 2µM $CuCl_2$, 2µM $NiCl_2$, 2µM Na_2MoO_4, 2µM Na_2SeO_3, 2µM H_3BO_3), and 50 µM $FeCl_3$. To increase the expression of protein from the plasmids the A600 was measured once an hour to determine the optimal time to induce with isopropyl ß-D-1-thiogalactopyranoside (IPTG) using a SpectraMax M3 Spectrophotometer. Protein overexpression was induced with 1mM IPTG once A600 was 0.6-0.8 and left to incubate for ~92hrs at 12°C and 250 rpm.

Round 2:

The second round of overexpression cultures was done with RIPL cells expressing 2 plasmids; Um145.His6.IF::pET28b and Um36.102.1::pET28b. Both overnight and day cultures were made using the same procedure and media conditions as mentioned previous. Day cultures were grown at 12°C, shaken at 210 rpm for 90 hrs. Proteins were extracted and His6-tagged protein was purified as described following, both extracts were quantified and visualized with SDS-PAGE. Samples were frozen with LN_2 and stored at -80°C until future use.

Round 3:

GST-tagged proteins were stockpiled as a precaution and to ensure enough enzyme to study. RIPL cells expressing GST.l.DMOXv2 and GST.l.DMOXv3 were each taken to make 2 cultures of each for overexpression. The culturing and induction were done in the same conditions following the same protocol as previous rounds of culturing to eliminate any variability. Cells were harvested and homogenized as described following, proteins were purified using a GSH-sefinose gravity-flow column also described following. Extracts were quantified, visualized with SDS-PAGE as described previously and stored at -80°C until future use.

Protein extraction

Cell harvesting

Day cultures were taken out of the incubation chamber following the >72 hr period. The 400 mL cultures were divided into two and centrifuged for 10 minutes at 10,000 x *g* at 4°C. Supernatants were discarded and pellets resuspended in 10% v/v glycerol then centrifuged as before. The last resuspension was done into 20 mL of each protein affinity tag's corresponding lysis buffer. GST.l.DMOXv2- and GST.l.DMOXv3- expressing cells were resuspended with 1x PBS (10 mM Na_2HPO_4 (pH 7.4), 150 mM NaCl, 2.7 mM KCl) and 10% v/v glycerol. STag.T.Um36.102.1 and Um36.102.1 were resuspended into STag wash/binding buffer (20 mM TRIS-HCl (pH 7.5), 50 mM NaCl, 0.01% v/v Triton X-100, 10% v/v glycerol) and Um36.102.1His6 into 50 mM sodium phosphate (pH 7.4), 300 mM A600, 10 mM imidazole, and 10% v/v glycerol. Resuspended cells were frozen in LN_2 and stored at -80°C.

Cell homogenization

Cells harvested as described previously were homogenized using the Avestin Emulsifix B-15 homogenizer according to manufacturers' protocols were lysed with 3 passes at 21,000 psi. Lysate was centrifuged for 20 min at 12000 x *g* and 4°C, supernatant was saved as it contains the soluble proteins, and precipitate was also saved as an insoluble fraction. Each sample was analyzed as described previously, with 10% or 12% (w/v) SDS-PAGE.

Plasmid cloning

Overnight DH10β cultures were used for two purposes: saving cultures *via* glycerol standards and extraction of cloned plasmids. The four plasmids present in individual cultures were STag.T.Um36.102.1::pET29b, Um36.102.1His6::pET28b, Um36.102.1::pBAD24 and Um36.102.1::pET28b. Glycerol stocks are used for storing transformed *E. coli* cultures for

extended periods of time at -80°C. Standards were made by taking 500 µL of the overnight culture, diluted with 500 µL 100% glycerol and mixed thoroughly before flash freezing in LN_2.

For plasmid extraction, the remaining overnight culture was pelleted in a stepwise manner, adding 1 mL of culture, centrifuging at 10000 x g for 4 min, and discarding supernatant. This was repeated until the entire culture has been combined. Plasmid extraction was carried out using the Monarch Plasmid DNA Miniprep Kit (NEB, T1010) and protocol. pDNA purity and concentration was determined with the A260/A280 ratio, where a ratio greater than 1.8 was considered pure. A260 and A280 was measured using a Nanodrop 1000 Spectrophotometer (Thermo Scientific) and associated software.

Protein Purification

Soluble fractions confirmed to be positive for the presence of desired tagged fusion proteins were attempted to be purified using protein tag affinity medias. This was the case for GST- and His_6-tagged protein extracts. The purification protocols are as follows.

GSH-sefinose

GST Sefinose Resin was obtained from Bio Basic Inc. (BSP031) and purification was carried out with the manufacturer's gravity-column protocol with some modifications. Soluble fractions of *E. coli* RIPL expressing GST.l.DMOXv2 and GST.l.DMOXv3 were passed over the column three times, saving the NBE for purification analysis with SDS-PAGE. Bound protein was washed with 10 column volumes of wash buffer (1X PBS, 10% v/v glycerol) and the pooled wash fraction saved for SDS-PAGE analysis. GST-tagged protein was eluted with 2 column volumes of elution buffer (50mM TRIS-Cl (pH 8), 100 µM NaCl, 10% v/v glycerol, 10 mM freshly added glutathione), collected in 500 µL fractions for protein quantification and SDS-PAGE. Columns were regenerated according to manufacturer instruction for future use.

Nuvia Ni^{2+}-affinity column

Nuvia IMAC Ni^{2+} charged resin was obtained from Biorad (7800880, LOT 64113898), and purification was carried out following a modified protocol from the manufacturer. Soluble protein extracts from RIPL cells expressing Um145I.F.His6 and Um36.102.1His6 were passed over the column three times, keeping NBE for analysis. Unbound proteins were washed off with 50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, 25 mM imidazole, and 10% v/v glycerol, saving fractions for future analysis until no protein was detected using a non-quantitative Bradford assay. Bound proteins were eluted with 50 mM sodium phosphate (pH 7.4), 300 mM NaCl, 500 mM imidazole and 10% v/v glycerol, and fractions were collected for future use and SDS-PAGE analysis.

Protein Desalting

Protein extracts were desalted from one buffer to another using various MWCO concentrators. Large volumes were desalted using Amicon Ulra-4 10K MWCO (Millipore-Sigma), centrifuged for 10 minutes at 5,000 x *g* and 4°C. After each centrifugation the target buffer was used to dilute the protein, the centrifugation and buffer addition was repeated until salt was <5% of the initial concentration. For small volumes, Nanosep (Pall) 10K MWCO tubes were used, centrifugation was done at 14,000 x *g,* 4°C for 12 min intervals. The same buffer addition-centrifugation cycle was implemented to the same effect.

Thrombin Cleavage of GST-tagged Protein

To confirm GST-tagged DMOX proteins had been correctly transcribed, thrombin was used to remove the GST tag at the encoded cleavage site within the linker region (.l.) between the GST and the DMOX peptides. The encoded linker region was deemed necessary for successful cleavage in past projects , as done by Hannah Gale (21). Before thrombin cleavage,

GST.l.DMOXv2 and GST.l.DMOXv3 extracts were concentrated and buffer exchanged using a Millipore Amicon Ultra-4 MWCO, centrifuged at 7,500 x *g* and 4°C for 5 minutes. This was done in a stepwise manner to avoid having soluble proteins precipitate. Extracts were taken from the GST-column elution buffer (50 mM TRIS-HCl (pH 8), 100 mM NaCl, 10% v/v glycerol, 10 mM glutathione), and exchanged into 1x PBS that is optimal for thrombin activity.

Trial 1

The first round of cultures for GST.l.DMOXv2 and GST.l.DMOXv3 were used in the initial thrombin cleavage trial. Thrombin (Biovision) was used in the cleavage trial using 30U of thrombin for every 1000 µg of GST-tagged protein. Protein (36 µg) was used in the cleavage trial, done at 20°C with end-over-end rotation. Protein (6 µg) was removed from the digest at 0, 1, 2, 4, and 6 hrs and added into prepared PBS and LBS to get to the final concentrations of 1x PBS, 1x LBS and 0.33 µg/µL protein. These solutions were denatured at 75°C for 5 min and then centrifuged at 10000 x g for 2 minutes, stored on ice directly following. Digest progression was analyzed using a 10% acrylamide gel and SDS-PAGE at 120 V for 1 hr 20 min. Gels were stained and analyzed as before.

Trial 2

Following the contamination seen in samples and buffers, degraded thrombin stocks from repeated freeze-thaw cycles are believed to have been the cause. New thrombin was purchased from Biovision, the cleavage trial protocol as seen above was repeated here. Samples in this trial were boiled instead of denatured at 75°C before being centrifuged at 10000 x *g* for 2 minutes in preparation for SDS-PAGE analysis.

Immunoblotting

Anti-GST immunoblot

Samples of protein across purification steps and expression trials were probed for the presence of GST-tagged proteins. Protein (2 μg for most samples, 400 ng for insoluble fractions) was resolved on a 10% (w/v) SDS-PAGE gel at 120V for 1.25 hrs. Pure GST (200 ng) was used as a positive control for the immunoblot. Gels were incubated in Towbin buffer (25 mM TRIS (pH 8.3), 192 mM glycine, 20% (v/v) methanol) for 15 min, with agitation at 4°C. Amersham Low-Fluorescence Polyvinylidene Fluoride (PVDF) membrane was activated by soaking in methanol (100%) for 15 seconds with agitation then rinsing for 2 minutes with MilliQ water. The PVDF membrane and Blotting Filter Paper (2.5 mm thick, 8.6 x 13.5 cm, BioRad) were soaked in Towbin buffer for 5 minutes, with agitation at 4°C. Towbin buffer was added to create a puddle on the lower electrode of a BioRad Semi-Dry Transblotter (BioRad #170-3940). The gel transferring system was assembled from the bottom and as follows; filter paper, PVDF membrane, gel, filter paper. Additional Towbin buffer was added between each layer and a glass pipette was used to roll out air bubbles. The top electrode and the lid of the transblotter were assembled. The transblotter was connected to a BioRad PowerPac. The current was 5.5 mA/cm^2 blot surface area and proteins were transferred for 32 min, with a voltage maximum of 15 V. Visible protein ladder was annotated using a LI-COR Biosciences WesternSure Pen on PDVF membrane for visualization by chemiluminescence. The membrane was blocked with blocking buffer of 5% (w/v) Amersham ECL Prime Blocking Reagent in 1X TRIS-buffered saline with Tween-20 (TBS-T) (50 mM TRIS (pH 7.5), 150 mM NaCl, 2.7 mM KCl, 0.1% (v/v) Tween-20) with agitation at 4°C overnight. The membranes were rinsed with TBS-T briefly for 3 x 5 minutes. The immunoblot was probed with primary antibody rabbit anti-GST IgG in a 1:1000

dilution with 2% (w/v) blocking buffer for 1.5 hrs at 20°C and washed the same as before. Secondary antibody probing was done with anti-rabbit IgG directly conjugated to horseradish peroxidase (HRP) at a 1:20,000 dilution in blocking buffer for 1 hour at room temperature with agitation. The immunoblots were washed 3 x 5 minutes with TBS-T. The immunoblot was developed using Cytiva Amersham ECL Select Western Blotting Detection Reagent. Immunoblots were incubated with 1 mL working reagent in the dark for 5 minutes. The immunoblot was imaged using a BioRad VersaDoc imaging system.

Blot stripping

Before reblotting the PVDF membrane, both primary and secondary antibodies were stripped off using the procedure from (33). The blotted PVDF membrane was placed into 20 mM TRIS-HCl (pH 7.5), 6 M guanidine hydrochloride, 0.2% (v/v) IPAGEL CA630 (Nonidet P40), and 100 mM β-mercaptoethanol, agitated gently at 20°C for 5 minutes, washed with 1x TBS 4 times, and repeated once more. The stripped membrane was equilibrated into 5% (w/v) blocking buffer for 30 minutes at 20°C.

Anti-DMOX immunoblot

The stripped and blocked membrane was washed 3 x 5 minutes with TBS-T. The membrane was probed with primary rabbit anti-DMOX IgG , made by Hannah Gale (21) in 2% blocking buffer at a 1:1000 dilution for 1 hr at 20°C, with gentle agitation. The blot was washed the same as before, probed with the secondary antibody of anti-rabbit IgG conjugated to HRP in a 1:20000 dilution in 2% blocking buffer for 1 hr at 20°C. Visualization was undertaken the same as described previously.

GST-tagged Protein Clean-up

One of the benefits of using GST-tagged proteins is the prospect of removing the tag being left with only pure untagged enzyme. This project implemented two procedures with the goal of cleaving and removing the GST from GST.l.DMOXv2/v3. The first procedure cleaved the GST-tagged proteins and then passed them over the GSH-sefinose column to do what. The second cleaved GST-tagged proteins bound to GSH-sefinose resin and washed away liberated DMOX protein, leaving bound GST.

Attempt 1

The GSH-sefinose column purified proteins previously buffer exchanged into 1X PBS were taken through a full thrombin cleavage and free GST removal. GST.l.DMOXv2 and GST.l.DMOXv3 (1 mg) were cleaved using 30 U of thrombin for 2 hrs at 20°C with rotation. Samples were placed on ice directly following cleavage. The cleaved proteins were passed over a GSH-sefinose column 3 times similarly to as described in the purification. In this case the NBE should contain pure l.DMOXv2 or v3, and remaining unbound protein was brought out with 2 mL of 1x PBS and 1M NaCl. Fractions of each were collected and concentration of protein was determined before saving a sample for SDS-PAGE analysis.

Attempt 2

With contamination seen within the buffers of previously purified proteins, uncontaminated extracts from round 3 of culturing, were used in another attempt to cleave and clean GST.l.DMOXv2 and GST.l.DMOXv3. The procedure was modified from what was described previously. Following the 2 hr thrombin cleavage of the whole extract, the sample was added to 60 µL of *p*-aminobenzamidine-agarose resin (Sigma, P1755), buffer exchanged into 1X PBS, and rotated at 4°C for 30 minutes. These solutions were centrifuged at 2500 x *g* and 4°C for

5 minutes, saving the thrombin-depleted supernatant. Samples at each stage of the purification were saved for analysis as described previously.

Attempt 3

Low pure protein yields led to another attempted cleavage and clean-up of the GST-tagged proteins. The second half of cultures from round 3 of cultures were used in an identical protocol as used in attempt 1. In this attempt fresh thrombin and GSH-sefinose resin was used. *P*-Aminobenzamidine-agarose resin to remove thrombin was excluded to retain more protein. Samples were saved and analyzed as described previously.

On-column cleavage

With the short-comings of previous clean-up attempts, a resin-bound batch protocol was used to be cleave and clean the GST.l.DMOXv2 and GST.l.DMOXv3 NBE samples from the 2nd round of culturing which appeared to have residual GST.l.DMOX protein. This protocol was adapted from (34). GSH-sefinose resin centrifuged at 700 x *g* and resuspended in 1x PBS, this was repeated 10X to ensure no remaining storage buffer. NBE fractions were combined and adsorbed to the resin at 20°C with rotation for 30 minutes. Unbound proteins were removed by centrifugation at 700 x *g,* removing supernatant, resuspending in 1x PBS and repeating 4 times. Resin-bound proteins now resuspended in 1x PBS with 30 U of thrombin and allowed to rotate at 20°C for 2 hr. Digested protein was centrifuged at 700 x *g* and supernatant containing free l.DMOX proteins was saved. The resin bound GST was washed twice by resuspending with 2 mL of PBS and centrifuging as before. Samples at each step of this process was saved and resolved by SDS-PAGE to monitor purity.

Coupled Enzyme Assay

NADH stock

In preparation for the final DMOX activity assay, each coupled enzyme assay needed to have their activities measured to adjust assay components. Possible oxidation of NADH stocks was quantified to confirm that the concentration of NADH was greater than the concentration of NAD without regard to reduction status. NADH quantitation measurements were done by taking an absorbance spectrum from 220-400 nm, detecting every 10 nm using a SpectraMax M3 spectrophotometer and comparing the absorbance at 260 nm and 340 nm with their corresponding extinction coefficients, ε_{260}= 16.9 mM^{-1} cm^{-1}, ε_{340}= 6.22 mM^{-1} cm^{-1} (35)

MDH Assay

The MDH activity was determined using the assay protocol from biochemica information (36), adjusted to a final volume of 800 µL. MDH (Sigma, M1567, porcine heart, ~6700 U/mL) was removed from storage buffer (ammonium sulfate) by centrifugation at 12000 x *g* 2 min 4C discarding the supernatant and resuspending into 100 mM TRIS-HCl at pH 8.0. The final assay solution was composed of 94 mM TRIS-HCl (pH 8.0,) 0.5 mM oxaloacetate, 0.2 mM NADH and 5 µL of MDH stock. The assay was kinetically monitored *via* absorbance at 340 nm (A340) using the SpectraMax M3 spectrophotometer using SoftMax Pro software. Activity was calculated from the change in A340 (ΔA340) with an extinction coefficient of 6.22 L/mmol/cm and a pathlength of 1 cm.

PEPC Assay

Corn shoot protein extracts known to have PEPC activity was used to confirm whether the PEP had degraded to an unusable state, this was done using the assay as described in the following but using 20 µL of crude corn shoot extract in place of pure PEPC (Dana Mills,

unpublished data). Following confirmation of PEP quality, multiple stocks of dry purified PEPC from corn (Calbiochem, 524810, *Z. mays,* 11 U/mg and 21 U/mg) and one isolated from *E. coli* (Sigma-Aldrich, P8079, inactive) were tested for enzymatic activity. PEPC was reconstituted into 100 mM TRIS-HCl buffer (pH 8.0) to get a dilution of 0.64-0.70 U/mL from the activity provided by the manufacturer. The final assay contained 57 mM TRIS-HCl (pH 8), 3.1 mM PEP, 9.7 mM Na_2CO_3, 9.7 mM $MgCl_2$, 0.14 mM NADH, 9.7 U/mL MDH. Activity was measured through the oxidation of NADH to NAD^+ through the ΔA340 as described previously, using the SpectraMax M3 and SoftMax Pro software.

LDH Assay

To test and re-evaluate the LDH activity (Sigma, L3916, rabbit muscle, 222 U/mL), a modified assay from what the manufacturer provided was performed. The LDH was desalted using previously described methods, from $(NH_4)_2SO_4$ into assay buffer. The assay volume was downsized from 3 mL to 800 µL and BSA was excluded from our assay preparations as there will be no BSA in the DMOX coupled-enzyme assay. The final assay was composed of 100 mM sodium phosphate (pH 7.5), 0.12 mM NADH, 1.1 mM pyruvate and ~0.6 U *L*-LDH. Activity was determined using ΔA340/min correcting to concentration with the extinction coefficient 6.22 mM/cm. Absorbance was measured using a SpectraMax M3 and presented in SoftMax Pro software.

DMOX activity assay

With the confirmation of each assay component being functional, a full DMOX activity assay was attempted, using a reduced volume assay in a 96-well opaque plate. There were 6 protein extracts used in duplicate to try and measure DMOX activity; Um145I.F crude soluble extract from Isaiah Baldwin, my crude Um36.102.1 extract, affinity-purified

STag.T.Um36.102.1 from Ca Nguyen, and my affinity-purified .l.DMOXv2, l.DMOXv3, Um36.102.1His6, and Um145.IF.His6. Each protein was desalted into 20 mM MOPS buffer (pH 8.0), except for Um36.102.1 which was in STag binding buffer (20 mM TRIS-HCl (pH 7.9), 200 mM NaCl, 0.2 mM DTT, 10% v/v glycerol and 0.01% Triton X-100). Each extract was incubated with 1 mM $FeSO_4$ for ~1hr before plate loading. The protein control was induced pET28b-NP crude soluble extract from Kate Dunning, buffer exchanged into MOPS buffer, using as similar desalting protocol as described previously using a Nanosep 10K MWCO (Pall, OD010C34). Each protein extract was compared against a sample of the same protein boiled for 5 mins as an activity control. To measure background activity due to any endogenous CO_2 present as HCO_3^-, there were wells that contained no DMSHB or no protein. Three Na_2HCO_3 concentrations (0.1 mM, 1 mM and 10 mM) were used to determine if the rest of the enzyme assay was functional, as this introduces HCO_3^-. An equimolar 25 mM MOPS/TRICINE buffer (pH 8.0) was degassed initially with a vacuum desiccator (~350mbar), however there was concern that the vacuum was not strong enough, thus the buffer was sparged with nitrogen gas for 30 minutes. Oxygen was reintroduced by bubbling air back into the buffer through a soda lime filter to remove CO_2. Final assay components are as follow, 25 mM MOPS/TRICINE (pH 8.0), 5 mM $MgCl_2$, 1U LDH, 2.11U MDH, 0.2 mM NADH, 2 mM PEP, 1 mM *D,L*-DMSHB (synthesized by Katie Blakeman, Vial #7), 25 mM neutralized ascorbic acid, and 0.45 U PEPC. The amount of protein loaded for each extract varied, 100 µg of Um36.102.1 and pET28b-NP, 19.5 µg of STag.T.Um36.102.1, 30 µg of Um36.102.1His6 and Um145.IF.His6, 7.5 µg .l.DMOXv2 and 3.75 µg .l.DMOXv3. Protein was delivered in 25 µL volumes and 75 µL assay master mix was added directly prior to detection. The progress of the assay was measured as the fluorometric loss of NADH to NAD^+ through excitation at 340 nm and emission at 460 nm, over

30 minutes with detection on the shortest interval using a SpectraMax M3 spectrophotometer and SoftMax Pro software.

RESULTS

Expression of Recombinant Proteins

Various forms of recombinant protein plasmids, both full-length DMSP synthase encoding all 3 enzymatic domains and DMOX alone were transformed in two strains of *E. coli,* RIPL and DH10β. DH10β were used to clone plasmids for future use. RIPL cells were used for overexpression of recombinant proteins with the intention of purifying the expressed proteins and performing DMOX activity assays. Each recombinant protein expressed in each strain of *E. coli* successfully, without being toxic towards the host, in overexpressing protein or cloning plasmids for future work. Carboxyl-terminal encoded His6- and N-terminal encoded Stag-tagged recombinant forms of Um36.102.1were observed to be expressed at the expected size (~145 kDa) (figure 7). While difficult to view, GST-tagged DMOX proteins were also expressed in these bacterial hosts close to expected size (~50 kDa) (figure 6)

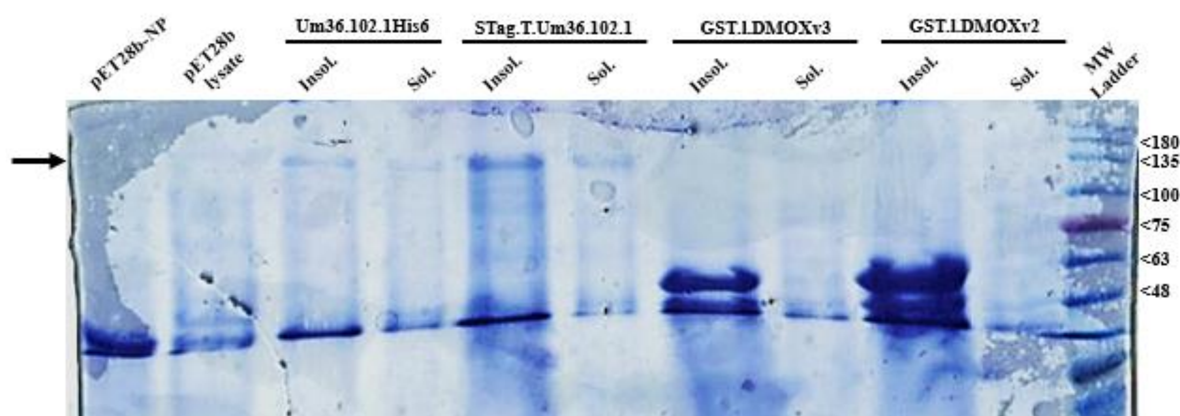

Figure 6. Expression of Um36.102.1His6, Stag.T.Um36.102.1, and GST.l.DMOX in *E. coli* RIPL. All cells harbouring plasmids were grown in LB-phosphate (pH 7.8), 50 µM $FeCl_3$, 1x trace metals with 50 µg/mL respective antibiotics (ampicillin or kanamycin) at 12°C shaken at 220 rpm for 92hrs, induced with 1 mM IPTG. Approximately 10 µg of protein was resolved on a 10% (w/v) SDS-PAGE gel at 120V for 1hr 23min, stained with Coomassie Brilliant Blue. Both Um36.102.1 recombinant forms were expected to be ~145 kDa, GST.l.DMOXv2/v3 were expected to be ~50 kDa. Size determined with the FroggaBio BLUeye prestained protein ladder.

The second round of protein overexpression in RIPL also showed successful expression of the C-terminally tagged Um145.His6.IF and the untagged Um36.102.1. Each recombinant protein was also soluble, which is required for future analysis and protocols. Um145.His6.IF and Um36.102.1 were observed to be expressed at the expected size (~145 kDa) (figure 8).

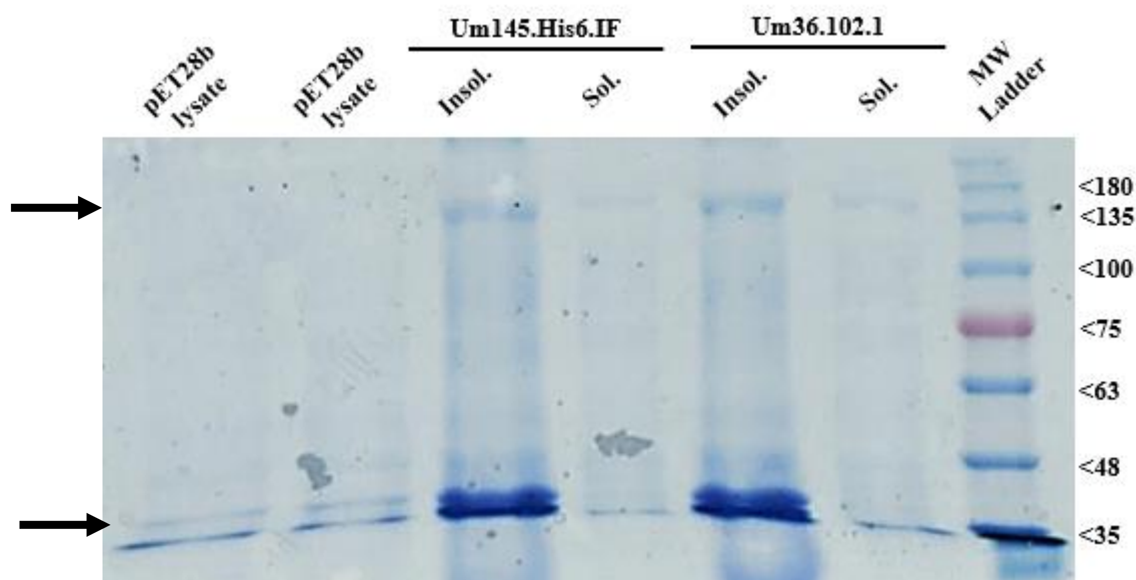

Figure 7. Expression of Um145.His6.IF and Um36.102.1 in Electrocompetent *E. coli* RIPL. Both recombinant proteins were expressed in RIPL grown in LB-phosphate (pH 7.8), 50 µM $FeCl_3$, 1x trace metals with respective antibiotics at 12°C shaken at 220 rpm for 90hrs, induced with 1 mM IPTG. Approximately 10 µg of protein was resolved on a 10% (w/v) SDS-PAGE gel at 120V for 1hr31min, stained with Coomassie Brilliant Blue. Both recombinant forms were expected to be ~150 kDa, as indicated by arrows. Size determined using the FroggaBio BLUeye prestained protein ladder.

Protein purification

The ability to purify and cleave GST tags from GST-tagged recombinant proteins is one of the features that makes them appealing for enzyme purification. The first step of this process was shown to be effective as each gravity GSH-affinity column purification showed a high degree of success in purifying the two recombinant forms here; GST.l.DMOXv2 and GST.l.DMOXv3 (figures 8-10). These recombinant forms were also observed at the anticipated size of ~50 kDa. There were bands of unidentified proteins seen throughout the gel but observed

best in figure 15, where there are proteins seen at ~89 kDa, ~70 kDa, and ~25 kDa. Purification resulted in ~1.5 mg of protein being isolated.

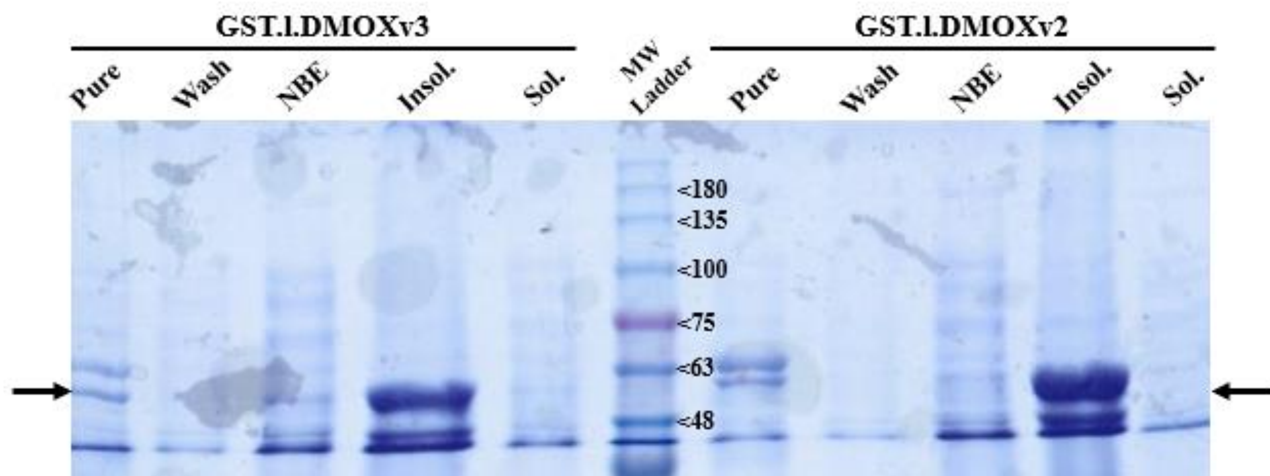

Figure 8. SDS-PAGE to monitor purification of GST.l.DMOXv2/v3 using a GSH-sefinose gravity column. Proteins expressed in RIPL cells, grown in LB-phosphate media (pH 7.8, 50 µM $FeCl_3$, 1x trace metals, 50 µg/mL ampicillin, shaken at 220 rpm, 12°C for 90hrs, induced with 1 mM IPTG). Purification was performed with a 1 mL column volume of BioBasic GSH-sefinose resin and eluted into 50 mM TRIS-HCl (pH 8.0), 50 mM GSH. Proteins (10 µg of soluble and insoluble fractions, and 5 µg of the other samples) were resolved on a 10 % (w/v) SDS-PAGE gel and stained with Coomassie Brilliant Blue. Pure proteins at the correct size are indicated by arrows. Unknown protein band at ~63 kDa.

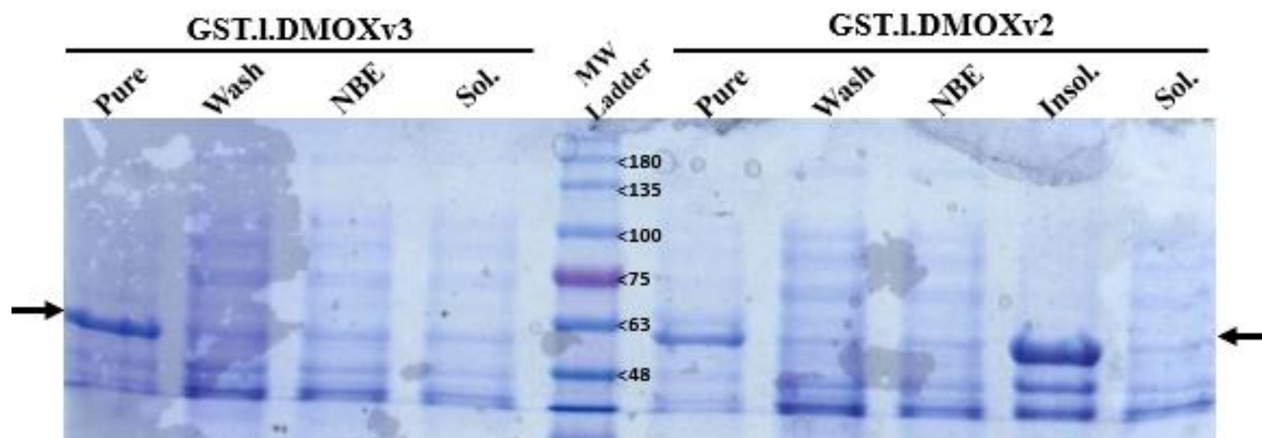

Figure 9. SDS-PAGE of GSH-sefinose column-purified GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins from the first extract of round 3 of culturing. Expressed in RIPL cells, grown in LB-phosphate (pH 7.8, 50 µM FeCl3, 1x Trace metals, 50 µg/mL ampicillin), induced with 1 mM IPTG, and shaken at 220 rpm, 12°C for 92 hrs. Proteins were purified with a 1 mL column volume of GSH-sefinose resin, eluting into 50 mM TRIS-HCl (pH 8.0), 100 µM NaCl, 10% v/v glycerol, 10 mM freshly added glutathione. Approximately 10 µg of each protein resolved on a 10% SDS-PAGE gel, stained with Coomassie Brilliant Blue. The MW ladder was FroggaBio BLUeye prestained protein ladder. Pure protein at the anticipated size denoted with arrows.

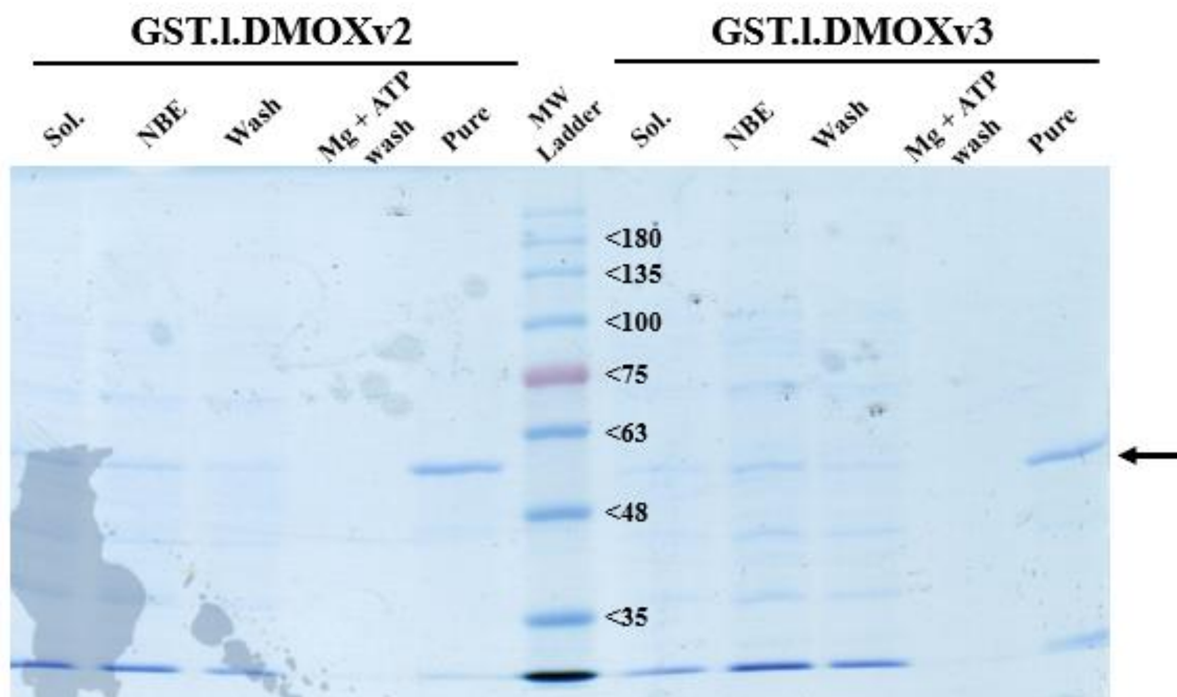

Figure 10. SDS-PAGE of GSH-sefinose column-purified GST.l.DMOXv2 and GST.l.DMOXv3 from the second extract of culturing round 3. Expressed in RIPL cells, grown in LB-phosphate (pH 7.8, 50 µM $FeCl_3$ 1x trace metals, 50 µg/mL ampicillin), induced with 1 mM IPTG, and shaken at 220 rpm, 12°C for 92 hrs. Proteins were purified with 1 mL column volume of GSH-sefinose as used in previous purifications, with the addition of an MgATP wash to remove chaperone proteins. Approximately 10 µg of each protein resolved on a 10% SDS-PAGE gel, stained with Coomassie Brilliant Blue. The MW ladder was FroggaBio BLUeye prestained protein ladder. Pure protein at the anticipated size denoted with arrows.

Purifications of C-terminally His6-tagged recombinant proteins purified with a Ni^{2+} affinity column were successful in isolating the recombinant protein of interest. Um36.102.1.His6 and Um145.His6.IF were expressed are shown purified at the size expected (~150 kDa) (figure 11). Multiple bands seen at smaller MWs (~75 and 95 kDa) were identified as degraded recombinant proteins due to an unforeseen sensitivity to denaturing at ~100°C when preparing samples for SDS-PAGE (data not shown). Future SDS-PAGE samples were denatured at 75°C as it was seen to nearly eliminate this observation, iodoacetamide blocking of sulfhydryl groups before denaturation had no effect (data not published). Purification resulted in ~1 mg of each protein.

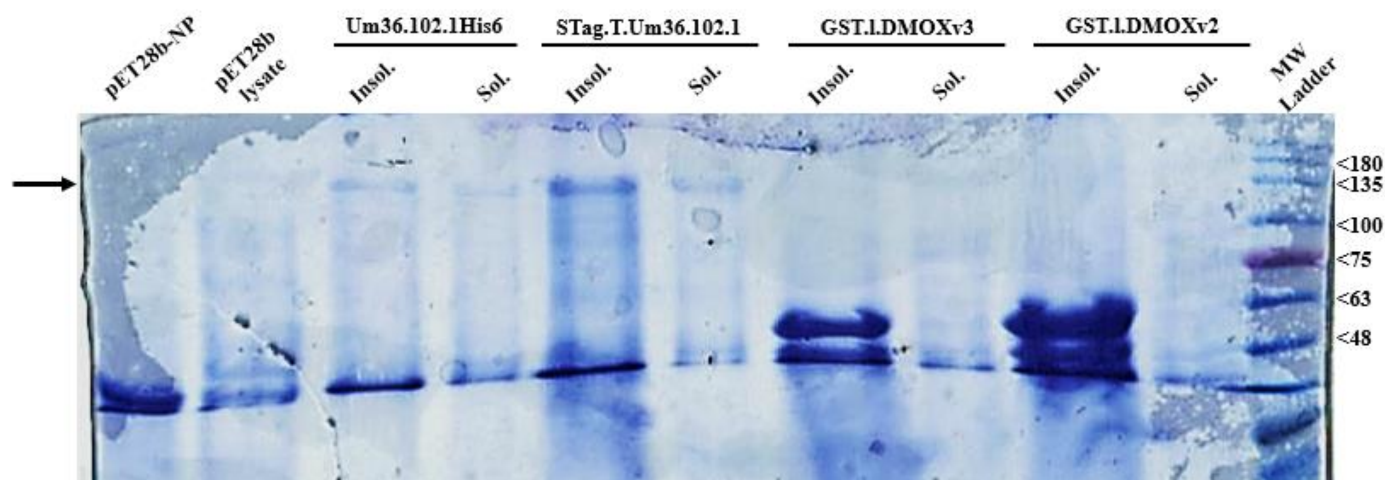

Figure 11. SDS-PAGE of Nuvia Ni^{2+}column purified Um145.His6.IF and Um36.102.1His6 recombinant proteins. Expressed in RIPL cells, grown in LB-phosphate buffer (pH 7.8, 50 µM $FeCl_3$, 1x trace metals, 50 µg/mL kanamycin), shaken at 220 rpm, 12°C for 90hrs, induced with 1 mM IPTG. Purification was performed with a 1 mL column volume of Nuvia IMAC Ni-charged resin. 10 µg of protein was resolved on a 10% (w/v) SDS-PAGE, and stained with Coomassie Brilliant Blue. The MW ladder was FroggaBio BLUeye prestained protein ladder. Proteins at the anticipated size denoted by the arrows.

Thrombin Cleavage trials

The ability of the GST-tagged recombinant proteins to be cleaved by thrombin showed mixed results. In trial 1 there was minimal observed cleavage, due to inactive thrombin, and what was able to be seen was not measurable due to the large band of contamination seen across the entirety of the gel (figure 12). After much delay caused by troubleshooting and investigation, this contamination was eventually determined to be from water contamination from the poorly maintained Gairdiner building reverse osmosis system which fed the Milli-Q water purification apparatus used to make electrophoresis and sample buffers. Consequently, anything made or stored in buffers made from the contaminated water was disposed of. Trial 2 showed much more promising results. The initial undigested protein is as seen in the 0 hr lane in figure 13, there are the proteins at the size as anticipated for the GST.l.DMOX recombinant forms. As the digestion progressed the full-size band disappeared with the appearance of two new bands at the expected

size for the GST-tag (~20 kDa) and l.DMOXv2 or l.DMOXv3 (~30 kDa). There is minimal difference from 2 hrs onwards so future digestions were carried out for 2 hrs.

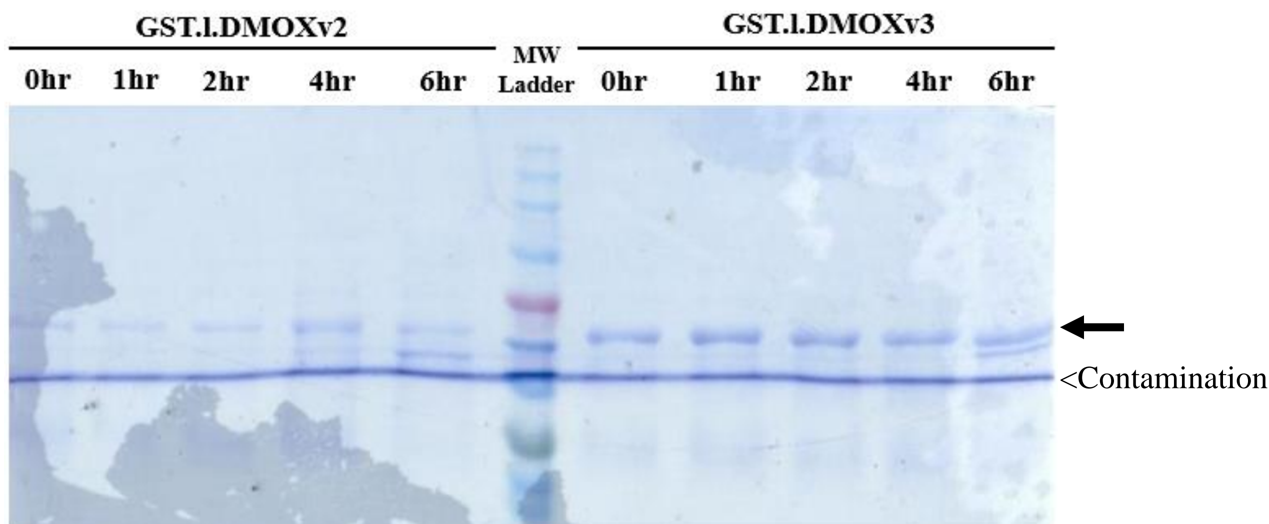

Figure 12. Thrombin Cleavage trial 1 of GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins. GST.l.DMOXv2/v3 were expressed RIPL cells and grown in LB-phosphate media (pH 7.8, 50 µM FeCl3, 1x Trace metals, 50 µg/mL ampicillin), induced with 1 mM IPTG, and shaken at 220 rpm, 12°C for 90 hrs. Proteins purified using GSH-sefinose resin and buffer-exchanged into 1X PBS were cleaved with 30 U thrombin per 1 mg protein, with samples taken at the times noted and boiled before loading. Protein (6 µg) was loaded into each well and resolved on a 10% (w/v) SDS-PAGE. Stained with Coomassie Brilliant Blue R250. The MW ladder was FroggaBio BLUeye prestained protein ladder. Large band of contamination is as seen, samples could not migrate past this point.

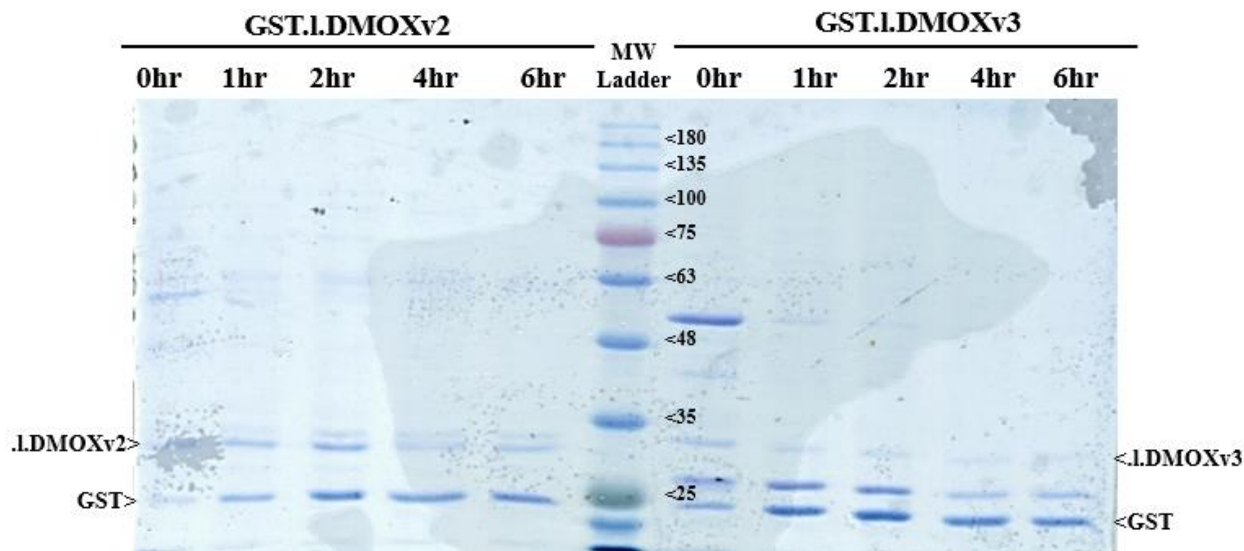

Figure 13. Thrombin cleavage trial 2 of GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins. GST.l.DMOXv2/v3 were expressed RIPL cells and grown in LB-phosphate media (pH 7.8, 50 µM $FeCl_3$, 1x Trace metals, 50 µg/mL ampicillin), induced with 1 mM IPTG, and shaken at 220 rpm, 12°C for 92 hrs. Proteins purified using GSH-sefinose resin and buffer exchanged into 1X PBS were cleaved with 30 U thrombin per 1 mg Protein, with samples taken at the times noted and boiled before loading. Protein (6 µg) was loaded into each well and resolved on a 12% (w/v) SDS-PAGE. Stained with Coomassie Brilliant Blue R250. Sized determined with the FroggaBio BLUeye prestained protein ladder.

Immunoblot of GST-tagged Recombinant Proteins

With the expression of recombinant proteins that appears to be the desired GST.l.DMOXv2 and GST.l.DMOXv3 products, the next step was to perform an immunoblot. The 10% (w/v) SDS-PAGE to confirm intact samples to utilize in an immunoblot was done and is as seen in figure 14. This showed that once again the cleavage appears to have been successful and that similar amounts of protein were loaded into each lane. These same samples were used in the immunoblots that were probed initially for GST, where it was observed that across the entirety of the progressive purification there is indeed GST incorporated into the expressed protein (figure 15) and is at a comparable size to the GST-control. The blot was stripped of antibodies and probed with a primary anti-DMOX antibody. This blot confirmed that the proteins expressed and purified have the region of the DMOX protein selected against by the antibody,

suggesting its identity as DMOX (figure 16). This improves the confidence in that the proteins expressed and purified are indeed the GST.l.DMOXv2 and GST.l.DMOXv3 recombinant proteins.

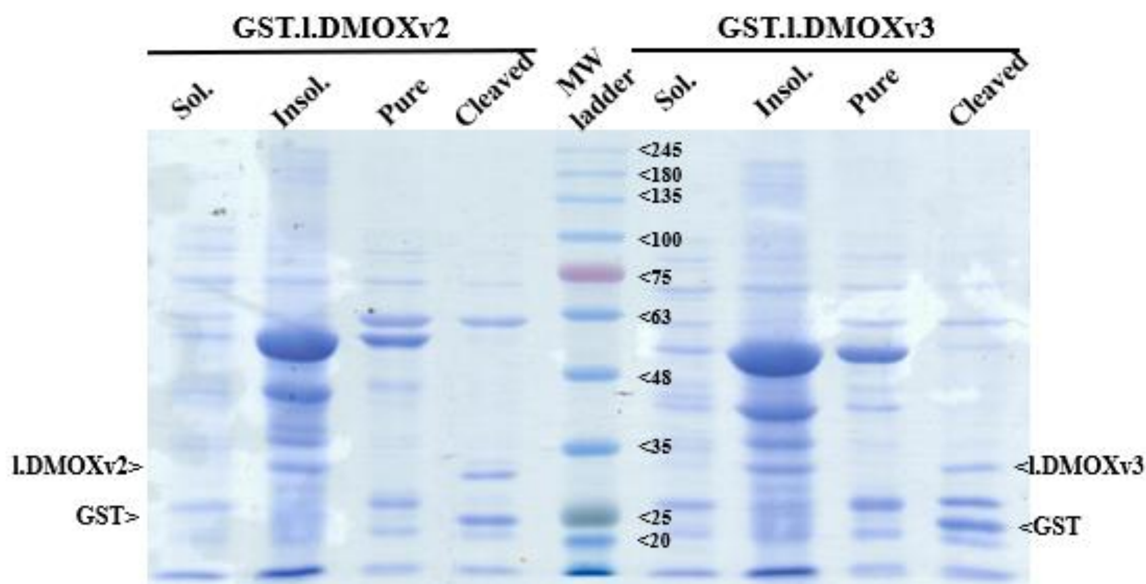

Figure 14. SDS-PAGE in preparation for immunoblotting to confirm protein identity of GST.l.DMOXv2/v3. GST.l.DMOXv2/v3 were expressed RIPL cells and grown in LB-phosphate media (pH 7.8, 50 µM $FeCl_3$, 1x trace metals, 50 µg/mL ampicillin), induced with 1 mM IPTG, and shaken at 220 rpm, 12°C for 92 hrs. Pure and cleaved sampled were desalted into 1X PBS for cleavage. Purification was done over 1 mL of GSH-sefinose resin, cleavage was carried out with 30 U thrombin per 1mg recombinant protein. Protein (5 µg) in each fraction was resolved on a 10% SDS-PAGE gel and stained with Coomassie Blue. The molecular weight ladder used was the FroggaBio BLUeye prestained protein ladder. Successful cleavage as indicated by the noted bands at the expected size of each component.

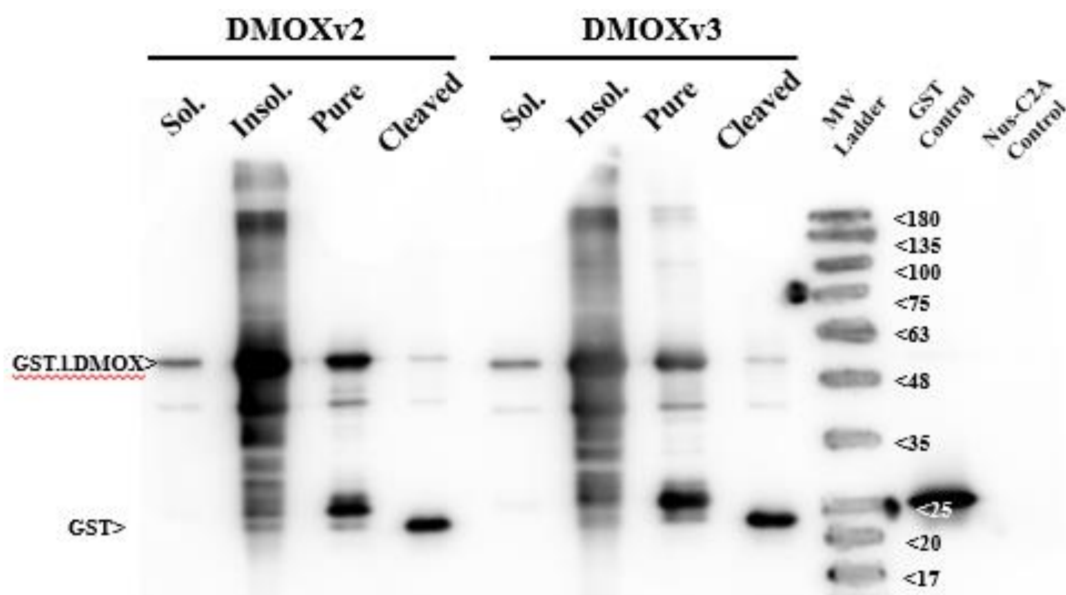

Figure 15. Immunoblot detection of GST tag in progressive purification and cleavage of GST.l.DMOXv2 and GST.l.DMOXv3. Proteins were expressed in RIPL cells as described previously. Purification was carried out over 1 mL column volume of GSH-sefinose resin, and cleavage was done with 30 U thrombin per 1mg recombinant protein. For the recombinant proteins, 2 µg of each sample were loaded, while 1 µg of the Nus-C2A control and 400 ng of the GST control were loaded and resolved on a 10% SDS-PAGE for 1hr 10min at 120V. Proteins were transferred to Amersham low-fluorescence PVDF membrane, the protein ladder was marked with a WesternSure pen to allow for visualization. The blot was probed with the primary rabbit anti-GST IgG, and probed with the secondary anti-(rabbit IgG) IgG conjugated to HRP. GE ECL reagents were used for imaging with the HRP reaction with a BioRad Versadoc imager. GST was ~24 kDa.

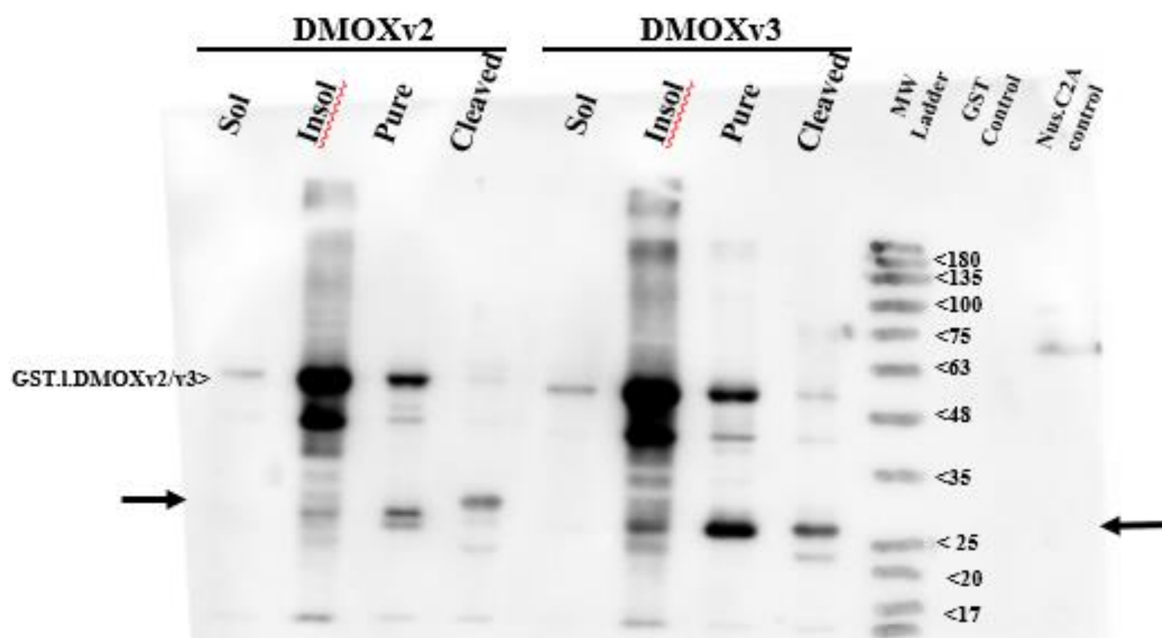

Figure 16. Immunoblot detection of DMOX in progressive purification and cleavage of GST.l.DMOXv2 and GST.l.DMOXv3. Proteins were expressed in RIPL cells as described previously. Purification was carried out over 1 mL column volume of GSH-sefinose resin, and cleavage was done with 30 U thrombin per 1mg recombinant protein. For the recombinant proteins, 2 µg of each sample were loaded, while 1 µg of the Nus-C2A control and 400 ng of the GST control were loaded and resolved on a 10% SDS-PAGE for 1hr 10min at 120V. Proteins were transferred to Amersham low-fluorescence PVDF membrane, the protein ladder was marked with a WesternSure pen to allow for visualization. The blot was probed with the primary rabbit Anti-DMOX IgG, and probed with the secondary anti-(rabbit IgG) IgG conjugated to HRP. GE ECL reagents were used for imaging with the HRP reaction. l.DMOXv2 was ~30 kDa, l.DMOXv3 was ~28 kDa, denoted with arrows.

GST clean-up

The first attempt to purify, thrombin cleave and clean the GST.l.DMOXv2/v3 recombinant proteins failed (gel not shown). Protein bands in a 10% (w/v) SDS-PAGE were faint and constant at one size, indicative of failed thrombin cleavage. Newly purchased thrombin was used in the following purification attempts. Thrombin cleavage was successful in trial 2, as the protein at ~50 kDa disappears following this cleavage and two bands appear at the size expected of GST and l.DMOXv2 or l.DMOXv3 (figure 17). Some level of purification was seen in GST.l.DMOXv3 as there is a protein retained throughout that is at the expected size of l.DMOXv3 (figure 18), this was determined by referencing other gels comparing the same samples with a ladder present and approximating protein size from those.

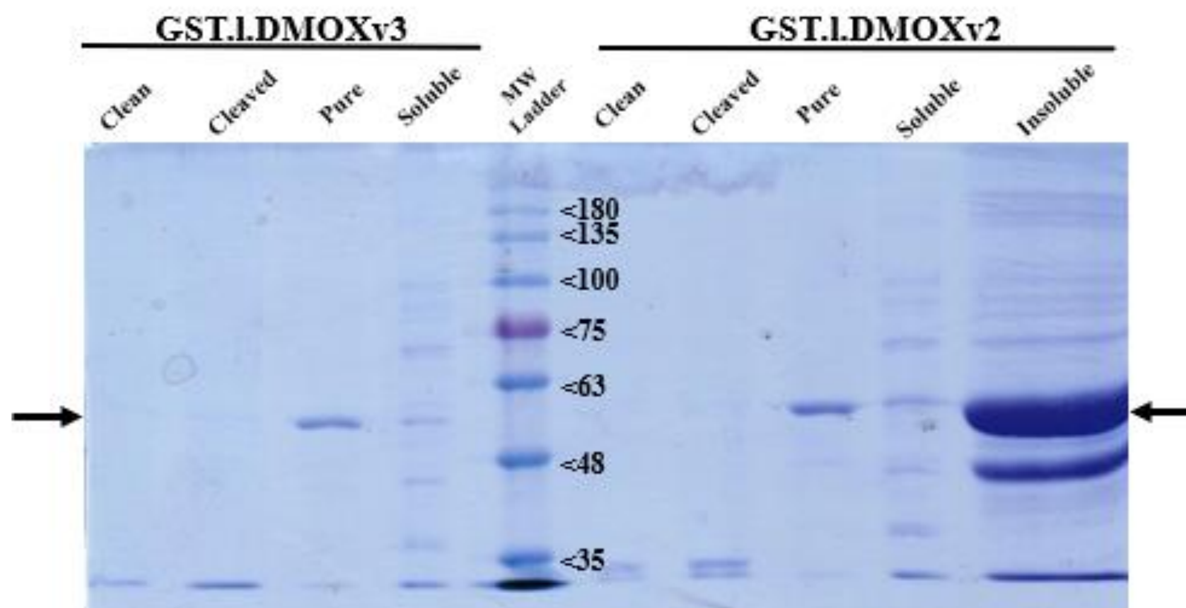

Figure 17. Progressive thrombin cleavage and clean-up attempt 2 of GST.l.DMOXv2 and GST.l.DMOXv3 extracts. Proteins were expressed in RIPL cells grown in LB-phosphate (pH 7.8, 50 µM $FeCl_3$, 1x trace metals, 50 µg/mL ampicillin) induced with 1 mM IPTG. Purified protein (~1mg) were cleaved with 30 U Thrombin, and then passed over a regenerated 1 mL GSH-sefinose column, the flow-through should contain l.DMOXv2/v3. Amount of protein loaded varied; soluble and insoluble at 10 µg, pure, cleaved and clean at 5 µg. Protein was resolved on a 10% SDS-PAGE gel, stained with Coomassie Blue. Acrylamide percentage not high enough to resolve small proteins, arrows denote GST.l.DMOXv2/v3. FroggaBio BLUeye prestained ladder was the molecular weight ladder of choice.

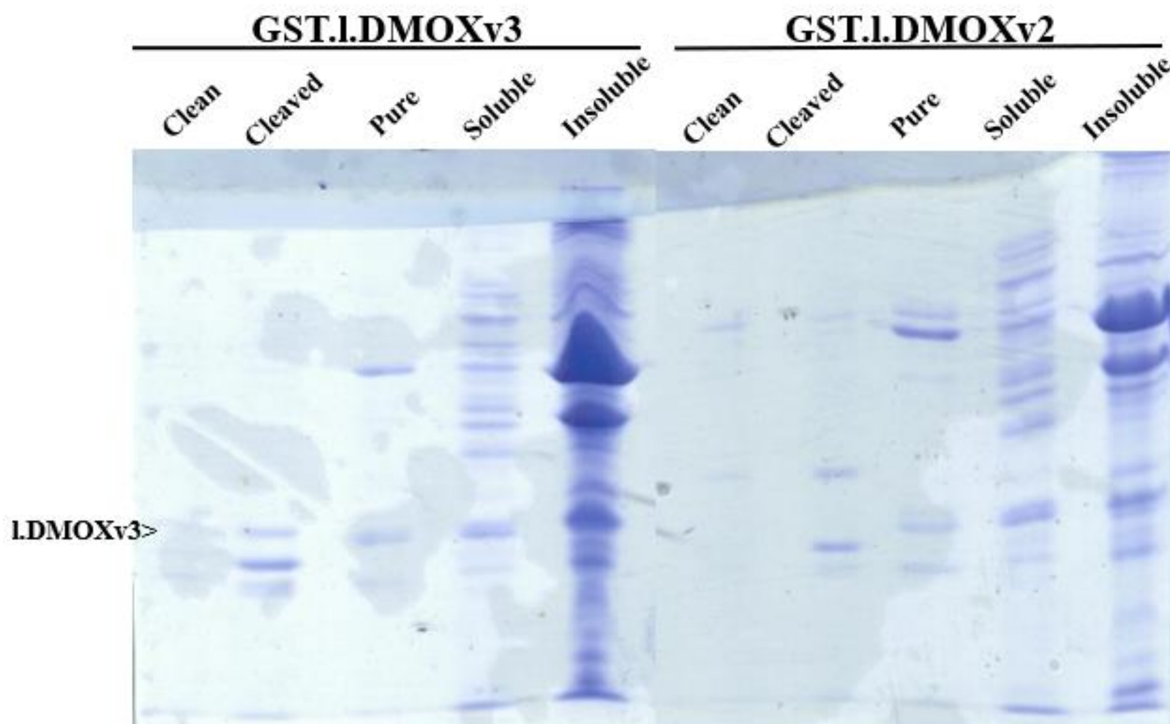

Figure 18. Progressive thrombin cleavage and clean-up attempt 2 of GST.l.DMOXv2 and GST.l.DMOXv3 extracts. Proteins were expressed in RIPL cells grown in LB-phosphate (pH 7.8, 50 µM FeCl3, 1x trace metals, 50 µg/mL ampicillin) induced with 1 mM IPTG. Purified protein (~1mg) were cleaved with 30 U thrombin, and then passed over a regenerated 1 mL GSH-sefinose column, the flow-through should contain l.DMOXv2/v3. Amount of protein loaded varied; soluble and insoluble at 10 µg, pure, cleaved, and clean at 5 µg. Protein was resolved on a 10% SDS-PAGE gel, stained with Coomassie Blue. Acrylamide percentage was not high enough to resolve small proteins, arrows denote GST.l.DMOXv2/v3. MW ladder was excluded by accident, banding comparison to past gels of the same samples were used to approximate size.

The resin-bound clean up attempted using the NBE's that appeared to have residual proteins at the size expected of GST.l.DMOX recombinant proteins (figures 10-11). These samples were combined and used in this attempt. The banding patterns are consistent across the clean-up process, thus there was both minimal binding to the GSH-sefinose resin and no observable thrombin cleavage (figure 19).

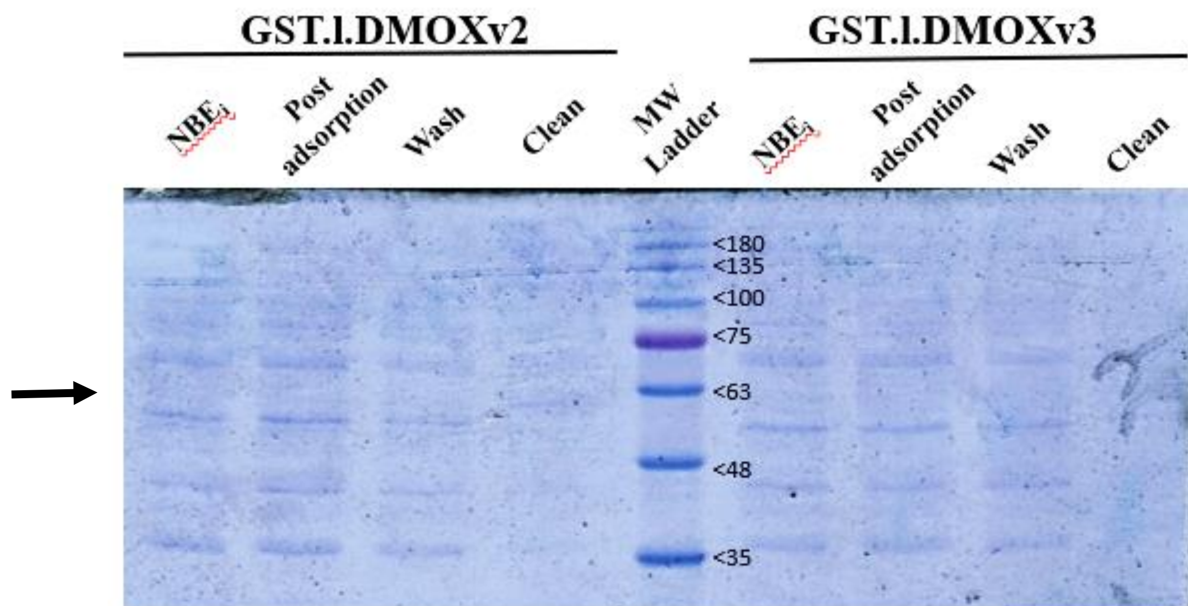

Figure 19. Batch method for clean-up of GST.l.DMOXv2 and GST.l.DMOXv3 NBEs with residual proteins at similar sizes. Proteins were expressed, grown purified as stated previously. NBE's of both cultures of v2 and v3 from round 3 were combined and desalted into 1X PBS. Samples were adsorbed to 1 mL of GSH-sefinose resin at 20° for 30 min while rotating, washed with PBS-T (1X PBS with 1% v/v Tween-20). Bound proteins were cleaved with 30 U thrombin for 2 hrs. Supernatant should contain untagged l.DMOXv2 or l.DMOXv3. Approximately 4 µg of protein was resolved on a 12% SDS-PAGE gel and stained with Coomassie Blue.

Work-up of DMOX Activity Assays

The stepwise work up procedure to validate the DMSHB oxidase components and potential were successful in taking CO_2 and oxidizing NADH. The MDH test assay on MDH isolated from bovine heart showed a high level of retained activity, at ~1000 U/mg. LDH from rabbit heart was tested individually and was active, at 222 U/mL. There were 3 isolates of commercial PEPC tested with a coupled assay to MDH and LDH: one from *E. coli* and two from *Z. mays.* The *E. coli* PEPC showed no activity. Isolated PEPC from *Z. mays* were both active, at 11 U/mg and 21 U/mg, where the more active isolate was carried forwards. The PEPC coupled assay to MDH and LDH highlights the ability of this assay to incorporate CO_2, as aqueous HCO_3^- into OAA with PEP. This suggests that this assay would be able to measure CO_2 produced by the DMSHB oxidase.

DMSHB Oxidase Activity Assay

The full coupled enzyme assay was not able to measure the activity of DMSHB oxidase, as NADH levels appeared to be depleted before the addition of any recombinant protein. NADH standards (0.2 mM) had a constant RFU (λ_{ex}= 340 nm, λ_{em}= 460) of ~1100. When the remaining assay components were added, with or without *D*-DMSHB, the RFUs were ~50. There was no linear slope, and thus no DMSHB activity could be measured. Potential sites of CO_2 contamination (e.g., ascorbate) were tested and when compared against degassed stocks of the same components and both showed identical results.

DISCUSSION

Each recombinant protein was able to be expressed and extracted readily from the bacterial host. The extracted proteins at each anticipated size were soluble and at the size expected for each. By culturing all the cells in similar conditions similar amounts of soluble proteins were extracted from each. The small variation and soluble state of the expressed proteins shows that the culturing conditions here can be used in the same manner for future studies of the recombinant proteins expressed here.

The two affinity-tagged recombinant protein were able to successfully isolated using their associated affinity resin. GST-tagged proteins were removed from the soluble fractions and were determined to have been ~1.5 mg, which was enough to continue experimentation. Purification of His6-tagged proteins were isolated in larger quantities, at ~3 mg which was enough to not warrant another investing in another round of cell culturing. Since both proteins were purified in reasonable quantities for the goals of this experiment, these procedures can be used in future attempts.

Thrombin cleavage trails showed mixed results. In early attempts to cleave the GST-tag from the recombinant proteins it was able to fully cleave the samples in 2 hrs, but when the same thrombin stocks were used in full scale cleavage for clean-up, they were ineffective. The freezing and thaw cycles most likely contributed to the poor cleavage results, by degrading the thrombin over time (37). This problem was solved with new thrombin being used.

Clean up attempts of GST.l.DMOXv2 and GST.l.DMOXv3 were tedious and showed variable results. The first attempt at cleaning up these proteins ended in failure, as cleavage was not successful. This was in part believed to be due to degraded thrombin, as the same extracts had been shown to be readily cleaved previously. In all cases there was a loss of overall protein quantity, with each passage over the GSH-sefinose column, which is expected with the initial purification but not when the second passage over the column should only be removing free GST. The GSH-sefinose purification column appears to retain protein within the resin, contributing to the reduced protein yield. Attempted resin-bound cleavage of NBEs showed that the proteins in the resin were not cleaved and proteins were seen at a larger size than even expected (38). This suggests that the residual proteins were most likely not GST.l.DMOXv2 or GST.l.DMOXv3. The issues at each step in the clean-up to get pure untagged DMOX protein, a primary benefit to using these proteins, may not be worth the effort for how tedious it became.

The attempted DMSHB oxidase coupled enzyme assay showed no activity in any of the samples tested. Results of the DMSHB oxidase assays were compared to NADH standards at the concentration the assay begins at. The RFUs of the assays were 5% of what was seen in the NADH standards, even without the presence of DMSHB oxidase proteins or *D*-DMSHB. This was determined to most likely due to CO_2 contamination from two potential sources. Protein extracts and some assay components could not be degassed effectively without risking the

integrity of the proteins or chemical component (39), thus there would be some level of CO_2 introduced to the final assay (40). Another possibility was with the reintroduction of O_2, even while removing CO_2 with soda lime. If there was even a small percentage of CO_2 that was not removed by the soda lime it would be significant as it was bubbled for 30 minutes. This method of reintroducing O_2 is one of the limitations of the coupled enzyme assay.

The nearly complete depletion of NADH in assays even without DMSHB oxidase is both positive and negative. On the positive side, the quick depletion of NADH with CO_2, either endogenous or contamination, shows that this coupled enzyme assay could be used to measure CO_2 production from decarboxylases with a high sensitivity (27, 28). With the amount of CO_2 that would have been left in the solution endogenously or through accidental contamination, the incorporation into PEP was efficient. The negatives were the obvious, DMSHB oxidase activity has yet to be measured directly and thus the method by which its activity is measured will need to be re-evaluated. The poor results of this assay attempt is indicative of some of the inherent difficulties of measuring the activity of this enzyme. In other coupled enzyme assays for measuring the production of CO_2, O_2 is rarely a substrate required for the assay to progress. Protocols for these types of decarboxylase assays are simpler since assay components/buffers can be degassed, removing CO_2, and used right away without reoxygenation steps.

FUTURE DIRECTIONS

With the success of recombinant protein expression, extraction and purification from the bacterial hosts, this mode of overexpression will continue to be used to make large amounts of these enzymes to study. The purified enzymes will continue to be used in DMOX activity assays, but the assays will be modified from what was used in this present study. The coupled enzyme assay used here can be redone within a more controlled environment to limit CO_2 contamination and O_2 can be reintroduced with a tank of pure oxygen. There are also alternative ways to reintroduce O_2, the first one to try would be degradation of H_2O_2 by catalase which can be used to saturate the buffers and any reactive oxygen species introduced can be scavenged by the ascorbate acid (41, 42). DMSP production could also be measured directly using methods requiring specialty derivatization dyes like PDAM and resolved by HPLC. PDAM in an acidic environment will react with produced DMSP and this new molecule can be separated and measured using HPLC, but this method is time and labor intensive (43). Direct measurement of DMSP, similar to what was done by Katy Dunning (22), could also be attempted, however this method is less sensitive. When a successful assay procedure is developed it will be used to fully characterize the pure form of this enzyme including determining K_m, V_{Max}, K_{cat}, pH optimum, and substrate specificity.

REFERENCES

1. Sievert, S., Kiene, R., and Schulz-Vogt, H. (2007) The Sulfur Cycle. *Oceanog.* **20**, 117–123
2. Anantharaman, K., Hausmann, B., Jungbluth, S. P., Kantor, R. S., Lavy, A., Warren, L. A., Rappé, M. S., Pester, M., Loy, A., Thomas, B. C., and Banfield, J. F. (2018) Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle. *ISME J*. **12**, 1715–1728
3. Howarth, R. W. (1984) The ecological significance of sulfur in the energy dynamics of salt marsh and coastal marine sediments. *Biogeochemistry*. **1**, 5–27
4. Gage, D. A., Rhodes, D., Nolte, K. D., Hicks, W. A., Leustek, T., Cooper, A. J. L., and Hanson, A. D. (1997) A new route for synthesis of dimethylsulphoniopropionate in marine algae. *Nature*. **387**, 891–894
5. Summers, P. S., Nolte, K. D., Cooper, A. J. L., Borgeas, H., Leustek, T., Rhodes, D., and Hanson, A. D. (1998) Identification and Stereospecificity of the First Three Enzymes of 3-Dimethylsulfoniopropionate Biosynthesis in a Chlorophyte Alga. *Plant Physiol*. **116**, 369–378
6. Dickschat, J. S., Rabe, P., and Citron, C. A. (2015) The chemical biology of dimethylsulfoniopropionate. *Org. Biomol. Chem.* **13**, 1954–1968
7. McNeil, S. D., Nuccio, M. L., and Hanson, A. D. (1999) Betaines and Related Osmoprotectants. Targets for Metabolic Engineering of Stress Resistance1. *Plant Physiology*. **120**, 945–949
8. Karsten, U., Kück, K., Vogt, C., and Kirst, G. O. (1996) Dimethylsulfoniopropionate Production in Phototrophic Organisms and its Physiological Functions as a Cryoprotectant. in *Biological and Environmental Chemistry of DMSP and Related Sulfonium Compounds* (Kiene, R. P., Visscher, P. T., Keller, M. D., and Kirst, G. O. eds), pp. 143–153, Springer US, Boston, MA, 10.1007/978-1-4613-0377-0_13
9. Sunda, W., Kieber, D. J., Kiene, R. P., and Huntsman, S. (2002) An antioxidant function for DMSP and DMS in marine algae. *Nature*. **418**, 317–320
10. Zhao, M., Xue, K., Wang, F., Liu, S., Bai, S., Sun, B., Zhou, J., and Yang, Y. (2014) Microbial mediation of biogeochemical cycles revealed by simulation of global changes with soil transplant and cropping. *ISME J*. **8**, 2045–2055
11. Charlson, R. J., Lovelock, J. E., Andreae, M. O., and Warren, S. G. (1987) Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. *Nature*. **326**, 655–661
12. Hill, R., White, B., Cottrell, M., and Dacey, J. (1998) Virus-mediated total release of dimethylsulfoniopropionate from marine phytoplankton: a potential climate process. *Aquat. Microb. Ecol.* **14**, 1–6
13. Kiene, R. P., Linn, L. J., and Bruton, J. A. (2000) New and important roles for DMSP in marine microbial communities. *Journal of Sea Research*. **43**, 209–224
14. Yoch, D. C. (2002) Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological Degradation to Dimethylsulfide. *Appl Environ Microbiol*. **68**, 5804–5815
15. Quinn, P. K., and Bates, T. S. (2011) The case against climate regulation via oceanic phytoplankton sulphur emissions. *Nature*. **480**, 51–56
16. Hanson, A. D., Rivoal, J., Paquet, L., and Gage, D. A. (1994) Biosynthesis of 3-Dimethylsulfoniopropionate in Wollastonia biflora (L.) DC. (Evidence That S-Methylmethionine Is an Intermediate). *Plant Physiol*. **105**, 103–110

17. Kocsis, M. G., and Hanson, A. D. (2000) Biochemical Evidence for Two Novel Enzymes in the Biosynthesis of 3-Dimethylsulfoniopropionate in Spartina alterniflora1. *Plant Physiology*. **123**, 1153–1162
18. Cody K. Harper (2017) *Purification, Characterization, and Immunoblot Analysis of Methionine Aminotransferase from the Ulva intestinalis Dimethylsulfoniopropionate Biosynthesis Pathway*. HBSc Thesis, Mount Allison University, Sackville NB
19. F. Hossain (2018) *Sensitivity, Specificity, and Application of Antibodies for Ulva species Methylthiohydroxybutyrate S-Methyltransferase from the Dimethylsulfoniopropionate Biosynthesis Pathway*. HBSc Thesis, Mount Allison University
20. K. Blakeman (2017) *Synthesis and Enzymatic Evaluation of Alternate Substrates for MTHB S- Methyltransferase in Ulva*. HBSc Thesis, Mount Allison Unversity
21. Hannah L. Gale (2019) *Specificity, Sensitivity, and Application of Antibodies Against Algal Dimethysulfoniohydroxybutyrate Oxidase*. HBSc Thesis, Mount Allison University
22. Katherine E. Dunning (2018) *Cloning and Enzymatic Characterization of Algal Dimethylsulfoniohydroxybutyrate Oxidase*. HBSc Thesis thesis, Mount Allison University
23. Dana Mills (2017) *Cloning and Expression Optimization of an Ulva mutabilis Methylthiooxobutyrate Reductase Gene Candidate*. HBSc Thesis, Mount Allison University
24. Lorentzen, E., Siebers, B., Hensel, R., and Pohl, E. (2005) Mechanism of the Schiff Base Forming Fructose-1,6-bisphosphate Aldolase: Structural Analysis of Reaction Intermediates. *Biochemistry*. **44**, 4222–4229
25. Fessner, W.-D., Schneider, A., Held, H., Sinerius, G., Walter, C., Hixon, M., and Schloss, J. V. (1996) The Mechanism of Class II, Metal-Dependent Aldolases. *Angew. Chem. Int. Ed. Engl.* **35**, 2219–2221
26. Pojer, F., Kahlich, R., Kammerer, B., Li, S.-M., and Heide, L. (2003) CloR, a Bifunctional Non-heme Iron Oxygenase Involved in Clorobiocin Biosynthesis *. *Journal of Biological Chemistry*. **278**, 30661–30668
27. Shen, W., McGath, M. K., Evande, R., and Berkowitz, D. B. (2005) A continuous spectrophotometric assay for human cystathionine beta-synthase. *Anal Biochem*. **342**, 103–110
28. Liao, C., Wang, Y., Tan, X., Sun, L., and Liu, S. (2015) Discovery of novel inhibitors of human S-adenosylmethionine decarboxylase based on in silico high-throughput screening and a non-radioactive enzymatic assay. *Sci Rep*. **5**, 10754
29. Sender, P. D., Martín, M. G., Peirú, S., and Magni, C. (2004) Characterization of an oxaloacetate decarboxylase that belongs to the malic enzyme family. *FEBS Letters*. **570**, 217–222
30. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. **72**, 248–254
31. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**, 680–685
32. *Lab FAQs: Find a Quick Solution* 3rd Ed., Rouche
33. Yeung, Y.-G., and Stanley, E. R. (2009) A solution for stripping antibodies from polyvinylidene fluoride immunoblots for multiple reprobing. *Anal Biochem*. **389**, 89–91
34. Guan, K. L., and Dixon, J. E. (1991) Eukaryotic proteins expressed in Escherichia coli: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal Biochem*. **192**, 262–267

35. Rex M. C. Dawson, Daphne C. Elliott, William H. Elliott, and Kenneth M. Jones (1986) *DATA FOR BIOCHEMICAL RESEARCH*, 3rd Ed., Oxford University Press, New York
36. *Biochemica Information* (1973) Boehringer Mannheim GmbH, West Germany
37. Cao, E., Chen, Y., Cui, Z., and Foster, P. R. (2003) Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. *Biotechnol Bioeng*. **82**, 684–690
38. Morales, E. S., Parcerisa, I. L., and Ceccarelli, E. A. (2019) A novel method for removing contaminant Hsp70 molecular chaperones from recombinant proteins. *Protein Sci*. **28**, 800–807
39. Zhang, L., Zhang, Y., Cheng, J., Wang, L., Wang, X., Zhang, M., Gao, Y., Hu, J., Zhang, X., Lü, J., Li, G., Tai, R., and Fang, H. (2017) Inert Gas Deactivates Protein Activity by Aggregation. *Sci Rep*. **7**, 10176
40. Lucile, F., Cézac, P., Contamine, F., Serin, J.-P., Houssin, D., and Arpentinier, P. (2012) Solubility of Carbon Dioxide in Water and Aqueous Solution Containing Sodium Hydroxide at Temperatures from (293.15 to 393.15) K and Pressure up to 5 MPa: Experimental Measurements. *J. Chem. Eng. Data*. **57**, 784–789
41. Beers, R. F., and Sizer, I. W. (1952) A SPECTROPHOTOMETRIC METHOD FOR MEASURING THE BREAKDOWN OF HYDROGEN PEROXIDE BY CATALASE. *Journal of Biological Chemistry*. **195**, 133–140
42. Ransy, C., Vaz, C., Lombès, A., and Bouillaud, F. (2020) Use of H2O2 to Cause Oxidative Stress, the Catalase Issue. *Int J Mol Sci*. **21**, 9149
43. Spielmeyer, A., Gebser, B., and Pohnert, G. (2011) Dimethylsulfide sources from microalgae: Improvement and application of a derivatization-based method for the determination of dimethylsulfoniopropionate and other zwitterionic osmolytes in phytoplankton. *Marine Chemistry*. **124**, 48–56