Characterization of virulence factors targeting *Apis mellifera*:
*Varroa* Toxic Protein and LarvinA

by

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ABSTRACT

CHARACTERIZATION OF VIRULENCE FACTORS TARGETING APIS MELLIFERA:
VARROA TOXIC PROTEIN AND LARVINA

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Apis mellifera populations are declining due to a variety of factors, including the parasitic mite, Varroa destructor, and the bacterial disease, American foulbrood (AFB). This thesis describes the biochemical characterization of two important virulence factors: LarvinA from AFB, and Varroa Toxic Protein (VTP) from the mite. LarvinA was kinetically characterized and confirmed to be a functional C3-like mono-ADP-ribosyltransferase toxin. N-terminal deletions and single-residue variants led to the identification of novel interactions between the α1-helix and the active-site of LarvinA, including the role of net charge in cell entry. Next, VTP was purified using glutathione-based affinity and size-exclusion chromatography. Analysis of the primary sequence and circular dichroism spectra revealed that VTP is primarily an α-helical protein; however, it is thermally labile based on the temperature analysis of the protein. These findings can be combined with future studies to elucidate novel therapeutics.
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I’d also like to thank my second-floor family, who are a constant source of support, friendship and joy. We have had some amazing and ridiculous times together, which I’ll always cherish. There are too many people to specifically name, but please know that you all contributed to making graduate school an amazing experience! Finally, I’d like to thank my family for all they’ve taught me and their continued support. I certainly wouldn’t have been able to do this without them.
DECLARATION OF WORK PERFORMED

It has been necessary to include certain experiments carried out by other scientists in order to properly describe the significance of these findings. All the work contained in this thesis is my own with the following exceptions: the LarvinA model and yeast cytotoxicity assay were carried out by O. Tremblay. The macrophage assays were conducted either in part or by K. Heney. Purification of LarvinA WT and variants was done with the considerable help of both I. Caines and M. McCarthy. Finally, M. McCarthy also tested the glycohydrolase activity of ΔY2-A30, ΔY2-K33 and ΔY2-W34, as well as the melting temperatures of ΔY2-D23, ΔY2-K25, ΔY2-D27 and ΔY2-A30.
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LIST OF ABBREVIATIONS

ε-NAD⁺ – Etheno-nicotinamide adenine dinucleotide
ADP – Adenosine diphosphate
AFB – American foulbrood
AMP – Antimicrobial peptide
ARF – ADP-ribosylating factor
ARTT – ADP-ribosylating turn-turn
BSA – Bovine serum albumin
cAMP – Cyclic adenosine monophosphate
CD – Circular dichroism
CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CT – Cholera toxin
CT – Cholera toxin
DAPI – 2-(4-amidinophenyl)-1H -indole-6-carboxamidine
DMEM – Dulbecco’s modified eagle media
DT – Diphtheria toxin
DT – Diphtheria toxin
DTT – Dithiothreitol
DWV – Deformed Wing virus
EDTA - Ethylenediaminetetraacetic acid
ERIC – Enterobacterial repetitive intergenic consensus
GDP – Guanosine diphosphate
GH – Glycohydrolase
GST – Glutathione S-transferase
GTP – Guanosine triphosphate
HPLC – high-performance liquid chromatography
IAPV – Israeli Acute Paralysis virus
IMAC – Immobilize-metal-affinity chromatography
IPTG – Isopropyl β- d-l-thiogalactopyranoside
LB – Luria broth
MALDI-TOF – Matric-assisted laser desorption/ionization – time-of-flight
mART – Mono-ADP-ribosyltransferase
NAD⁺ - Nicotinamide adenine dinucleotide
OD – Optical density
PBS – Phosphate-buffered saline
PCR – Polymerase chain reaction
PFA – Paraformaldehyde
PMSF – phenylmethylsulfonyl fluoride
SD-Leu – Synthetic defined medium without leucine
SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis
UV – Ultra-violet
VTP – Varroa Toxic Protein
WT – Wild-type
Chapter 1: Introduction
1.1 Honey bee Diseases and Parasites

Apis mellifera, the Western honey bee, is a major contributor to global food stocks (1). Since many crops require animal pollination in order to sexually reproduce, pollinators are essential to crop production (1, 2). Honey bees play an especially important role, due to the wide variety of crops and plants they visit (1). In total, 35% of consumable food is reliant on bee pollination, and without it, many food products would decrease in quality and yield (1). Crop yields can be reduced as much as 90% in extreme cases, and in 2009, it was estimated that insect pollination contributed $212 billion globally (1, 2). As a result, honey bee populations are closely monitored, and the health of the hive is heavily managed.

Despite their adaptability to new environments, A. mellifera are targets of many parasites and diseases (2). These pathogens cause immunocompetence in their hosts, sometimes even using the defenses of the bee to their benefit (3). Honey bees are susceptible to various mites such as Acarapis woodi and Varroa destructor, viruses like Deformed Wing Virus (DWV) or Israeli Acute Paralysis Virus (IAPV), and bacterial infections caused by Melissococcus plutonius or Paenibacillus larvae (2). While any of these pathogens can negatively impact a colony, American foulbrood disease caused by P. larvae and the mite V. destructor are considered the most detrimental to hive health (1). Part of the success of these organisms is that they both use specialized virulence factors in their pathogenicity to target key host systems (Figure 1.1) (4, 5).
Figure 1.1: Virulence factors secreted by the honey bee pathogens, *Paenibacillus larvae* and *Varroa destructor* (5, 6).
1.2 American Foulbrood

American foulbrood (AFB) is a bacterial disease responsible for the loss of millions of hives worldwide \((2, 5, 7)\). It is a lethal, highly contagious infection that is best contained by burning all contaminated hives and materials and autoclaving all equipment \((2)\). Today, this remains the most viable method of controlling AFB infections, causing major losses to beekeepers worldwide \((2)\). The causative agent is a Gram-positive, spore-forming bacterium, known as \textit{Paenibacillus larvae} \((2, 5, 7)\). This pathogen targets the hive brood killing the colony progeny, which may lead to colony collapse \((7–9)\). Upon host nutrient depletion, the bacteria sporulate, producing billions of infectious spores \((9)\). These spores are spread within the colony and to neighbouring hives by nurse bees and foragers performing robbing behaviour, which leads to a rapid propagation of the AFB infection \((9)\).

Successful infection requires the larval consumption of only 10 bacterial spores \((9)\). During the initial stage, the bacteria proliferate in the midgut where they live commensally and feed on larval food \((5, 7, 9)\). In the second stage of the infection, \textit{P. larvae} releases exotoxins that target the cell-cell and cell-matrix junctions of the peritrophic matrix within the larval gut \((5, 7, 9)\). Matrix and underlying epithelial tissue degradation allow access to the hemocoel, or main body cavity of the larva, where the bacteria consume the remaining larval tissue. Upon resource depletion, the bacteria sporulate and the larval carcass is reduced to a ropey-mass \((5, 7, 9)\). The body then begins to dry and form a hard, scale-like structure that adheres to the bottom of the brood cell \((7, 9)\). Nurse bees will attempt to sanitize the cell by removing the scale; however, this activity causes the release of millions of spores and propagates the infection \((7)\). While older larvae possess a sufficiently developed immune system to control the disease, larvae under 36 hours old are susceptible to the
spores (5, 7, 9, 10). Once contracted, the bacteria will take 7-12 days to kill the host, depending on the genotype of \textit{P. larvae} (5, 7, 8).

1.2.1 Genotypes

The species \textit{P. larvae} can be subdivided into four genotypes based on differences in enterobacterial repetitive intergenic consensus (ERIC) sequences (5, 7, 8, 11). These sequences are repeating palindromic elements within the genomes of gut-dwelling bacteria, such as \textit{Escherichia coli} and \textit{Salmonella enterica}, that can be used as tools for identification of bacterial species and strains (8, 12). Employing ERIC-specific primers in repetitive-element PCR gives rise to distinctive banding patterns on agarose gels, allowing for bacterial recognition (8). Using this technique, \textit{P. larvae} genotypes ERIC I-IV were established after years of misclassification as separate species, and later as subspecies of \textit{P. larvae} (8).

Misclassifications of \textit{P. larvae} arose due to differences in colony morphology, energy metabolism, and toxin production between the genotypes (5, 11). They also differ in prevalence, with ERIC I and II being frequently isolated from AFB-infected hives, whereas ERIC III and IV could only be described from reference strains (7, 9). Genomic studies have therefore focused on the first two genotypes to characterize \textit{P. larvae} pathogenicity (5). While both are biologically active, the two vary significantly from one another (5). ERIC I produces white, smooth colonies and takes approximately 12 days to kill infected larvae, while ERIC II produces orange, convoluted colonies and takes only 7 days (9, 11). Bioassays have proven that ERIC II-IV all kill during the 7-day timespan, making ERIC I a more chronic AFB pathogen (9). This difference illustrates varying pathogenicity and virulence between \textit{P. larvae} genotypes (5).
The toxin loci within ERIC I and II were compared in an effort to explain the differences in virulence (5). Genomic analysis yielded many potential toxin-forming gene clusters, some resembling those from other pathogenic bacteria such as *Clostridium botulinum*. It is likely that these were acquired through horizontal gene transfer; however, most were inactive due to accumulated mutations. Surprisingly, ERIC I contains more functional toxin-encoding genes than the more virulent ERIC II. Five putative toxins were described, named Plx1-5, that showed similarity to the AB-toxin family. Functionally, this family of toxins utilizes two proteins that work in concert to attack the host cell. The A-domain typically contains the enzymatic activity, while the B-domain acts as a transporter across the cell membrane. Further investigation revealed that four of the five toxins were ADP-ribosyltransferase toxins, belonging to either the C2- or C3-subgroups (5).

1.3 Mono-ADP-ribosyltransferase toxins

Pathogenic bacteria confer damage to host cells through the secretion of virulence factors (13). These are often enzymes that target vital host proteins and disrupt their functions through covalent modifications (14, 15). Mono-ADP-ribosyltransferase (mART) toxins are a family of AB-toxins secreted by various pathogens, such as *Vibrio cholera*, *C. botulinum*, and *Corynebacterium diphtheria* (13, 16). ADP-ribosylation involves the transfer of an ADP-ribose moiety from a donor NAD\(^+\) molecule to a target macromolecule, resulting in altered cellular function (13, 14, 17). These enzymes typically modify nucleophilic residues of G-type proteins including Rho proteins, small trimeric G proteins, and actin (13–15, 17). When the target macromolecule is absent, mARTs may exhibit glycohydrolase (GH) activity, where the ADP-ribose moiety is transferred to a water molecule (14, 17). This family of enzymes is characterized by low sequence identity; however,
members can be classified through domain organization, target macromolecule and target residue (15).

1.3.1 Classification of mono-ADP-ribosyltransferase toxins

Sequence identity between mART toxins can be as low as 15% (15). Classification into this family, therefore, requires the comparison of several conserved catalytic domains and structural motifs (13, 15). Differences between the catalytic domains and motifs led to the creation of two distinct groups: the Diphtheria toxin (DT) group and the Cholera toxin (CT) group; however, for the purpose of this work only the CT group will be discussed (13, 15). The first motif of interest is found on the β1-strand, where a catalytic Arg residue contributes to NAD\(^+\) substrate binding (Figure 1.2) (15). The second motif is found on the β3-strand, which is characteristically followed by an α-helix. Here, a Ser-Thr-Ser motif participates in NAD\(^+\) positioning within the substrate binding pocket (15). Finally, an ADP-ribosylating turn-turn (ARTT) motif containing a conserved Glu residue contributes to the catalytic transferase activity (14, 15).

CT-toxins are classified as AB-toxins, but due to variance in structure and macromolecular target, the group has been subdivided into CT-, C2-, and C3-like toxins (13, 15). CT-toxins consist of a 28 kDa catalytic A-subunit non-covalently bound to five, 12 kDa domain-translocating B-subunits (13). The B-subunits bind to GM1 ganglioside and the toxin is internalized via the GM1 ganglioside receptor. ADP-ribosylating factor (ARF) then binds and activates the A-subunit; which in turn targets G\(_s\), the alpha-subunit of the heterotrimeric G-protein. This modification maintains G\(_s\) in an active state, disrupting cAMP signaling and leads to ion-channel efflux. The C2-subclass functions as classical AB-toxins, where the A-subunit is 50 kDa and the B-subunit is 80 kDa. These proteins are independently synthesized, and associate at the membrane of the target
cell (13). Once internalized, these toxins will covalently modify monomeric G-actin at Arg177 leading to deregulation and the ultimate collapse of actin filaments (13, 14). The primary catalytic activity is housed within an E-X-E motif, characteristic to this subgroup (14). Finally, the C3-like group are single domain toxins, consisting solely of a catalytic A-subunit of 25 kDa (13, 15). These toxins target intracellular Rho-GTPases using a catalytic Q-X-E motif; however the mechanism used by C3-toxins to gain entry into host cells is not well understood (13–15). Rho-GTPases mediate actin association, such as stress fibril formation and actomyosin contraction, through GTP (active) or GDP (inactive) bound states (18). ADP-ribosylation of Rho proteins causes a shift in the affinity of the protein for its effectors (13). Specifically, Rho-GTPases become sequestered in the cytoplasm due to an increased affinity towards Rho-guanine nucleotide dissociation inhibitor. This inhibition of Rho-signaling results in deregulation of actin filaments leading to cytoskeletal collapse. These modifications drastically impact overall cellular function, making this enzyme-class useful tools in bacterial infection (13).

Structural analysis of the catalytic A-protein of C2-toxins revealed two distinct ADP-ribosylation active sites (13, 19). These sites divided the protein into two structurally homologous segments, referred to as the N- and C-domains (19). This topology is thought to have resulted from a gene duplication event, but only the C-domain active site has remained functional. The N-domain has evolved into an adapter region responsible for associating with the translocating B-protein of the C2-subgroup. Interestingly, the catalytic C-domain shares over 30% homology to C3-like toxins, suggesting the two groups share common ancestry (19). C2-like toxins share structural homology with C3-like toxins, displaying a characteristic mixed α/β-fold where a five-stranded β-sheet (β1, β2, β4, β7 and β8) packed against a second, three-stranded β-sheet (β3, β5, and β6)
forms the core of the protein (14, 17). Alpha-helices structurally surround the core, with four α-helices (α1-4) at the N-terminus and two additional helices (α5a and α5) connecting the β1- and β2-strands. It is the cleft between the two β-sheets and helical bundles that forms the NAD⁺-binding site (14, 17).

Loop regions on the protein contribute to substrate binding and recognition (14, 17). The first is a phosphate-nicotinamide (PN) loop which connects the two β-sheets through the β3- and β4-strands (14, 17). The normally flexible PN-loop becomes more ordered upon NAD⁺-binding, forming a more compact binding-cleft (14). Residues found on this loop also contribute to the orientation of the NAD⁺ molecule. In both the C2- and C3-subgroups, a conserved arginine residue hydrogen bonds with a phosphate within the NAD⁺ substrate, and an aromatic residue packs against the nicotinamide ring; however, some toxins have been found without this aromatic residue (14). The ADP-ribosylating turn-turn (ARTT) loop of C2- and C3-toxins connects the β5- and β6-strands and is responsible for substrate specificity (14, 17). This loop houses either the E-X-E or Q-X-E motif responsible for the transferase activity of the enzyme (14). In C3-toxins, the ARTT loop has also been proven to interact with hydrophobic patches on the RhoA substrate through a conserved aromatic residue (20).
**Figure 1.2: C3-toxin topology.** C3bot1 (PDB 1UZI) shown in cartoon with helices in lightblue, \(\beta\)-strands in palegreen and loops in bluewhite, with the exception of the ARTT-loop shown in purple and the PN-loop shown in raspberry. Using the structural alignment tool in PyMOL v1.3, the NAD\(^+\) substrate (cyan) was modelled into the active site using the C3bot1-NAD\(^+\) complex (PDB ID: IGFZ.A) as a template. All residues are shown as spheres and coloured by element. Structural annotation (A), location of the NAD\(^+\)-binding pocket (B), and location of the three catalytic motifs (C).
1.4 Mono-ADP-ribosyltransferase toxins from P. larvae: Plx2A and C3larvin

Potential toxins within the ERIC I and II genomes have been investigated in silico using known virulence-encoding genes as reference (5). Five putative toxins were identified in ERIC I, named Plx1-5 (5, 7). All proteins showed similarity to AB-toxins, with Plx1 and 2 predicted to have ADP-ribosylation activity (5, 7). Interestingly, the A-subunit of Plx2, denoted Plx2A, shared sequence homology with C3-like mART toxins (5, 7, 21). Unlike other members of the C3 family, Plx2 also encoded for a B-subunit (Plx2B), which shared homology with the translocating domains of C2-like toxins (5). Another exoenzyme-producing gene locus was found in both ERIC I and II, named Txy7 and TxyIII, respectively (5, 7). The product was predicted to be another binary AB-toxin, with the A-subunit encoding for a C3-like protein later named C3larvin (5, 7, 16). The B-subunit, however, had acquired numerous mutations which implied it was non-functional (5, 7). Both enzymes vary from the typical mART family classification of C3- and C2-toxins due to differences in domain organization and catalytic activity.

Despite the discovery of translocating B-subunits, both Plx2A and C3larvin behave as C3 exoenzymes. As such, both proteins target RhoA with $K_M$ values of $14 \pm 4$ μM and $17 \pm 3$ μM, respectively. Note that in both cases, human RhoA was used to characterize the transferase activity, due to the high sequence similarity between RhoA homologs; although it is assumed that the biological target of these enzymes would be the bee homolog, Rho1. In addition to the transferase activity, these enzymes also displayed GH activity. Although not biologically relevant, it serves as a useful tool for characterizing NAD$^+$ substrate binding and affinity (16, 21). Characterization of this reaction used an analog of NAD$^+$, known as etheno-NAD$^+$ ($\varepsilon$-NAD$^+$), which fluoresces upon
scission of the C-N glycosidic bond (16, 21). This assay revealed $K_M$ values of $120 \pm 16 \, \mu M$, and $176 \pm 7 \, \mu M$, for C3larvin and Plx2A, respectively. Finally, both enzymes were tested against the mouse macrophage cell line, J774A.1, to determine if they could permeate the cell membrane. Macrophage cells treated with nanomolar concentrations of Plx2A showed a distinct elongated morphology, resulting from cytoskeletal dysregulation. Conversely, cells treated with C3larvin showed no difference in morphology.

While Plx2A caused an altered morphology, proving it functioned without the B-domain, C3larvin failed to infect target cells (16, 21). With the associated B-domain deemed non-functional, it was hypothesized that C3larvin was meant to behave as a C3-exotoxin. Sequence comparison between C3larvin and other C3-like toxins then revealed a truncation in the N-terminal of helix 1 (16). It was hypothesized that the N-terminus contributed to C3 cell entry, based on the role of the N-domain in C2 toxins (16, 19). To test this theory, researchers created a chimeric protein using a 17-residue addition to the N-terminus of C3larvin from C3bot1, a well-characterized C3-toxin (16). The chimeric protein was fully functional and caused a morphology change to macrophage cells, supporting the literature (16). However, the inability of C3larvin to enter cells, coupled with the non-functional B-domain, meant that C3larvin could not play a role in ERIC I and II virulence. This was later confirmed in knock-out mutants of *P. larvae* (Julia Ebeling, pers. comm.).

### 1.5 Novel mono-ADP-ribosyltransferase toxin from *P. larvae*: LarvinA

Recently, another mART-encoding toxin locus was found within the ERIC III genotype (22). The encoded protein was identical to that of C3larvin, apart from an additional 58 residues on the N-terminus and a single substitution from Lys to a Glu residue at position 3 of C3larvin.
Additionally, the first 26 residues within the 58-residue addition were predicted to be a secretion signal. The presence of a secretion signal in the second toxin but not in C3larvin could indicate that the ERIC I and II genes have evolved, resulting in a non-functional product. Interestingly, this novel mART toxin was also found to have an associated B-domain similar to those found for Plx2A and C3larvin. As such, this protein has been named LarvinA, and will be one of the two virulence factors discussed in this work.

1.6 *Varroa destructor*

*Varroa destructor* is an ectoparasite that feeds upon honey bee tissues (1, 23). Originally from Asia, the native host of this mite is the Asian honey bee, *Apis cerana*. However, within the past 60 years, this mite has expanded its territory to nearly every corner of the globe due to its shift in host to the European honey bee, *Apis mellifera*. While the details of the expansion are unclear, it is likely that *A. mellifera* colonies were imported into affected regions, where the mite colonized within the new hives (1, 23). Now, it is assumed that any colony in an affected region will contain *V. destructor* (1). This recently established host-parasite relationship has left *A. mellifera* populations struggling, since the species lacks the co-adaptations to the mite seen in *A. cerana* populations (24). As a result, *V. destructor* infestation constitutes a major health concern for *A. mellifera* colonies.

*V. destructor* feeds on bee tissues and is, therefore, dependent upon its host for the entirety of its lifespan (1, 23). While it had been widely accepted that *Varroa* fed on haemolymph, recent evidence suggests that the mite sustains itself on the fat body of its host (25). Fat body is a vital organ for the honey bee, and in many ways can be compared to the mammalian liver. It participates in many cellular processes, including nutrient storage, detoxification, and immune function, that
contribute to the health of the bee at each stage of life (25). The typical target for feeding is the adult honey bee; however, during *V. destructor* reproduction, the mite instead targets the larval stage of the bee (1, 23). Prior to capping, the stage of larval development where the brood cell is sealed for pupation, the female mite will crawl inside the cell and bury into the larval food (23). This is thought to avoid the hygienic behaviour of nurse bees, and increases the likelihood of reproductive success (23). The mite is then released as the larva ingests the rest of the food, after which she lays her eggs (23). After hatching, the mother and offspring will then feed on the developing pupae, often causing long-term damage to the bee (1, 23). Parasitized bees are typically 7% smaller after emerging than their nest-mates and are shown to have shorter lifespans – both of which could be due to the loss of fat body at a critical stage in life (23, 25). Additionally, mites act as vectors for many viruses, increasing the frequency of infection and causing further harm to the bee (1, 23).

Viral infections predate the establishment of *V. destructor* colonization (23). Originally viewed as a minor threat to colony health, these infections have since been exacerbated by the introduction of the mite (23). Of the 18 known honey bee viruses, five are transmitted by *V. destructor* and there is a positive correlation between mite population and viral loads (1, 23). One virus, known as the Deformed Wing Virus (DWV), replicates within the mite’s salivary glands, elevating the viral particle titer delivered to the host upon mite feeding (1, 23). Moreover, symptoms of this disease, characterized by deformed wings and a shortened body, are more pronounced in parasitized individuals (23, 26). The relationship between viral loads, mite populations and honeybee health are multi-factorial and complex. However, the overall
deterioration in the health of parasitized individuals has led many to propose that mite saliva contains immunosuppressive agents (27, 28).

1.6.1 **Immunosuppressive agents and virulence factors within *Varroa* saliva**

While the effects of *V. destructor* parasitism have been investigated, identifying the components responsible has been a challenge (29). Such small mites produce only minute amounts of saliva, which has impeded the characterization of its components (29). Recently, researchers were able to identify proteins from the saliva using nano-liquid chromatography and tandem-mass spectrometry (6). This collection of over 400 proteins has been named the secretome and consists of proteins from the *Varroa* mite, *Apis mellifera* and the virus, DWV. From the identified proteins, it is clear that the secretome has a multitude of functions— including nutrient absorption, antimicrobial properties, and even resisting oxidative stress. Of specific interest, however, were the enzymes classified as potential virulence factors (6).

Certain proteins identified within the saliva of the *Varroa* mite have been previously characterized as virulence factors in other pathogenic bacteria and parasites (6). For example, calreticulin has been shown to increase infectivity of the protozoan parasite *Trypanosoma cruzi* due to its ability to interact with, and consequently inactivate, the host immune system (6, 30). Lysophospholipase has been implicated in the development of hepatic fibrosis caused by the parasitic infection of *Clonorchis sinensis* (6, 31). Enolase was also present in the saliva, and is a proposed virulence factor to various pathogenic bacteria, including *Paenibacillus larvae* (6, 32). Within this list of virulence factors, researchers also noted a novel protein, later named *Varroa* Toxic Protein (VTP) (33).
1.7 Varroa Toxic Protein

VTP is 14.7 kDa and has a predicted signal peptide sequence for secretion (33). It has a single homolog, neuroligin-4 from the predatory mite *Metaseiulus occidentalis*, and shares 29% sequence identity (33). Neuroligins typically bind calcium ions, implying that VTP could be a metalloprotease (33, 34). Recombinant VTP was purified from *Escherichia coli* Transetta cells and tested in both *A. cerana* and *A. mellifera* (33). *A. cerana* larvae showed a significant increase in mortality rates, whereas the mortality rate of *A. mellifera* larvae remained unchanged. It may seem counterintuitive that the native host to this parasite has increased mortality rates, but larval death likely protects the colony since it interferes with *Varroa* reproduction and therefore controls the mite population. It has been documented that *Varroa* mites avoid the worker brood in *A. cerana* hives and instead prefer the drone brood. This avoidance strategy is not seen in *A. mellifera* colonies, leading to parasitized worker bees and a weakened colony. Interestingly, adults and drone larvae from both species showed no increase in mortality when injected with VTP, suggesting an immunity to the protein (33).

The lethality of VTP to *A. cerana* worker larvae was not shared with *A. mellifera*; however, it did demonstrate another manner of toxicity against the European honeybee (33). Titers of the DWV virus were monitored between the various treatment groups, and only *A. mellifera* worker larvae showed increased levels of DWV. After pupation, 40-60% of the resulting adults had deformed wings, similar to larvae parasitized by *Varroa* or injected with crude *Varroa* lysate. This data suggests a relationship between VTP and DWV titers, implicating VTP as an immunosuppressive agent (33). Therefore, VTP represents a novel putative virulence factor and will be the second protein discussed in this work.
1.8 Research Rationale and Objectives

Parasites and bacterial infections pose a notable threat to *Apis mellifera* populations. Specifically, the bacterium *Paenibacillus larvae*, the causative agent of American foulbrood disease, and the mite, *Varroa destructor*, are considered particularly detrimental. These organisms utilize virulence factors in their pathogenicity to target key cellular systems within the host. Characterization of these virulence factors could, therefore, lead to a better understanding of these pathogens and how they convey damage to the host. This work will focus on the biochemical characterization of LarvinA and VTP.

1.8.1 LarvinA

LarvinA is proposed to be a mono-ADP-ribosyltransferase toxin. Classification of these diverse exotoxins has historically considered the topology and target substrate to differentiate each member; however, previously described mART toxins from *P. larvae* do not fit the classical groups. These hybrid exotoxins offer unique insight into this class of proteins and could show evolutionary ties between subgroups. Additionally, the similarity between LarvinA and the previously characterized C3larvin toxin presents a novel opportunity to investigate the role of helix I in the function of C3 toxins. This research could, therefore, offer insight not only into *P. larvae* infection, but into other bacterial infections that utilize C3 exotoxins. As such, this research will focus on characterizing the catalytic activity and cytotoxic effects of LarvinA, with specific interest in the role of the N-terminus.
1.8.2 Varroa Toxic Protein

Varroa mite parasitism shows a severe effect on *Apis mellifera* populations. It has negatively impacted colony vitality while simultaneously increasing the probability of viral infections. The mechanism by which *Varroa* accomplishes this is still not clear, or fully understood. Therefore, investigating the role of VTP as a virulence factor could significantly contribute to our understanding of Varroa parasitism. This research will focus on the biochemical characterization of this protein, with the intention of better understanding its structure and function.
Chapter 2: Materials and Methods
2.1 Protein Purification

2.1.1 Expression and purification of LarvinA

The larvinA gene was cloned into a pET-28a+ vector with an N-terminal poly-histidine tag. This vector was used to transform chemo-competent *E. coli* BL21 λDE3 cells through the heat-shock method. The cells were grown for 16 h on LB agar plates containing 30 µg/mL kanamycin at 37°C. These colonies were then used to inoculate 4 l of 2xYT media in the presence of 30 µg/mL kanamycin at 37°C. Protein expression was induced at an OD$_{600}$ of 0.6 by the addition of 1 mM of IPTG (final concentration) and proceeded for 3 h at 37°C. Finally, cells were harvested by centrifugation at 3,315 x g for 15 min at 4°C.

The cell pellet was resuspended in buffer containing 500 mM NaCl and 50 mM Tris-HCl, pH 7.5. The cells were lysed using an Emulsiflex-C3 high-pressure homogenizer (Avestin Inc., Ottawa, Canada) in the presence of 120 µM PMSF, 50 µg/mL CHAPS, 1 mM EDTA and 100 µg/mL DNase. Next, the homogenate was centrifuged at 23,700 x g for 55 min at 4°C to remove insoluble cell debris. The supernatant was then incubated with 10 mM MgCl$_2$ for 30 min at 4°C. This solution was passed over a Ni$^{2+}$-charged Chelating Fast Flow(TM) Sepharose column which was then washed twice, first with lysis buffer containing 25 mM imidazole, and secondly with lysis buffer containing 40 mM imidazole. A final wash with lysis buffer containing 250 mM imidazole was used to elute LarvinA. Fractions were analyzed with SDS-PAGE, and those fractions showing positive bands for LarvinA were dialyzed overnight in lysis buffer and further purified using a HiLoad 16/60 Superdex 200 column (GE Healthcare, Illinois, USA) in size-exclusion chromatography. Fractions showing pure protein after being analyzed via SDS-PAGE
were pooled, concentrated at 1509 x g using an Amicon Ultra-15, regenerated cellulose unit with a molecular-weight-cut-off of 10 kDa (Millipore, Germany) before being stored at -80°C.

2.1.2 Expression and purification of RhoA

*E. coli* BL21 λDE3 cells were transformed and cultured, and protein expression was induced as previously described (16). The cell pellet was resuspended in lysis buffer containing 2.5 mM MgCl₂, 150 mM NaCl, 1 mM DTT and 10 mM HEPES pH 7.5. Cells were lysed and the soluble fraction was separated as previously described for LarvinA; however, no EDTA was added. Next, supernatant was passed over a Glutathione Sepharose 4 Fast-Flow resin column (GE Healthcare, Illinois, USA) four times. The column was then washed with lysis buffer before the protein was eluted using 10 mM reduced glutathione. Eluted protein was assessed using SDS-PAGE and dialyzed overnight in lysis buffer. When necessary, samples were further purified using size-exclusion chromatography as described for LarvinA. Pure protein was concentrated and stored as previously described.

2.1.3 Expression and purification of Varroa Toxic Protein

The *vtp* gene was cloned into a pGEX-2T-TEV vector to enable fusion with an N-terminal glutathione-S-transferase (GST) tag. Cells were transformed and cultured as described for RhoA (16). Protein expression was induced as described, and proceeded for 16 h at 16°C, before being harvested as above.

The cell pellet was resuspended in 1x PBS buffer containing 1 mM DTT and 2.5 mM MgCl₂, pH 7.4. Cells were lysed and cellular debris was removed as described for RhoA. The soluble fraction was passed over Glutathione Sepharose 4 Fast-Flow resin column as above and
washed as described. All fractions were analyzed using SDS-PAGE, and fractions containing VTP-GST were pooled and dialyzed in 1x PBS containing 1 mM DTT and 2.5 mM MgCl₂, pH 7.4, for 16 h at 4°C. Samples were further purified using size-exclusion chromatography, concentrated and then stored as described.

2.2 Assessing Folded Stability

2.2.1 Circular dichroism spectroscopy

A JASCO J-815 CD spectropolarimeter was used to acquire the CD spectra of the protein by scanning through 250–190 nm 9 times and taking an average. Readings were taken at 25°C in a 1 mm pathlength quartz cuvette with a protein concentration of 0.16 mg/mL. LarvinA experiments were carried out in 10 mM Tris-HCl, pH 7.5 and 250 mM NaF, and VTP experiments were carried out in 1x PBS buffer, pH 7.4.

2.2.2 Melt curve

The thermal stability of each protein was assessed with a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, USA) using a final protein concentration of 0.5 mg/mL and 1x of SYPRO™ Orange (Invitrogen, Massachusetts, USA). LarvinA was measured in 500 mM NaCl and 50 mM Tris-HCl, pH 7.5, and VTP was measured in 1x PBS buffer containing 1 mM DTT and 2.5 mM MgCl₂, pH 7.4.

2.3 LarvinA Homology Model

The LarvinA homology model was created using the online server Phyre2 (Protein Homology/AnalogY Recognition Engine, V2.0) (35). Briefly, Phyre2 selects templates based on sequence homology, and builds possible models through secondary and tertiary structure
predictions (35). The Plx2A structure (PBD: 5URP, resolved to 1.65 Å) was used as a template, and the resulting homology model was reported with 100% confidence. Plx2A shares 55% sequence identity with LarvinA, and was chosen over C3larvin (99% sequence identity) to more accurately model the extended N-terminus of LarvinA.

2.4 Catalytic Activity of LarvinA

2.4.1 NAD$^+$-binding

The affinity between LarvinA and NAD$^+$ substrate was assessed using β-NAD$^+$ in a tryptophan-quenching assay. Measurements were taken using a Cary Eclipse fluorescence spectrometer (Varian Instruments, Mississauga, Canada) with an excitation wavelength of 295 nm, emission wavelength of 340 nm and excitation and emission bandpasses of 5 nm. A solution of 1.25 μM LarvinA in an initial volume of 600 μL buffer (20 mM Tris, pH 7.9, 50 mM NaCl) was titrated to achieve a range of β-NAD$^+$ concentrations between 1 and 1000 μM. Measurements were done in triplicate using 0.5 x 0.5 cm fluorescence quartz cuvettes.

2.4.2 Glycohydrolase activity

The glycohydrolase activity of LarvinA against ε-NAD$^+$ was measured on a Cary Eclipse fluorescence spectrometer (Varian Instruments, Mississauga, Canada) with an excitation wavelength of 305 nm, emission wavelength of 405 nm and excitation and emission bandpasses of 5 nm. The reaction was kept at a constant temperature of 25°C with a LarvinA concentration of 20 μM and ε-NAD$^+$ concentrations ranging from 0 – 500 μM in reaction buffer (50 mM NaCl and 20 mM Tris, pH 7.9). The reaction was monitored for 5 minutes, and the resulting slope was converted from fluorescence units to product concentration using a standard ε-AMP curve. All kinetics values were calculated using GraphPad.
2.4.3 Identifying RhoA as a target substrate

The ability of LarvinA to target RhoA was assessed using a fluorescein-NAD\(^+\) blot. RhoA, fluorescein-NAD\(^+\) and LarvinA were combined to final concentrations of 40 μM, 25 μM and 1 μM, respectively, in reaction buffer (5 mM MgCl\(_2\), 150 mM NaCl and 20 mM Tris-HCl, pH 7.4). This reaction was incubated for 1 h at room temperature with spinning while being protected from light. A positive control containing 1 μM of Plx2A, and a negative control without enzyme were also prepared. The reactions were stopped through the addition of Laemmli buffer and separated on SDS-PAGE. The gel was then imaged using the Trans-UV setting of a ChemiDoc MP (BioRad, Hercules, California).

2.4.4 Transferase activity

The ADP-ribosylation activity of LarvinA against RhoA was measured using an indirect, end-point assay. This was required due to the insolubility of RhoA, which precipitates after modification. The secondary product, nicotinamide, which is formed after the hydrolysis of NAD\(^+\), can instead be monitored to give the rate of the reaction. A final concentration of 300 μM β-NAD\(^+\) was used to ensure saturation, and RhoA-GST was varied between 0 – 150 μM in transferase buffer (5 mM MgCl\(_2\), 150 mM NaCl and 20 mM Tris-HCl, pH 7.4). The reaction was started through the addition of 1 μM LarvinA and allowed to continue for 5 min. It was then stopped by introducing a surplus of mobile phase solution (5% acetonitrile and 95% 20 mM monobasic phosphate buffer, pH 5.5) including an internal standard (2.5 μg/mL 4-nitrobenzoic acid). This was done to achieve a final ratio of 25% reaction to 75% mobile phase (v/v). This solution was passed through a Captiva filtration 96-well plate (Agilent Technologies, Santa Clara, USA) using a vacuum to remove all protein matter before being injected onto the Zorbax RX-C18, 4.6 x 250 mm, 5 μm diameter
An isocratic elution using the mobile phase listed above then separated the reaction components. The resulting nicotinamide peak area was calibrated by divided it by the peak area of 4-nitrobenzoic acid (internal standard). These values were calculated using the integration mode in Origin Pro version 8 (Northampton, MA). The calibrated nicotinamide peak formed from the blank run, containing only LarvinA and β-NAD$^+$, was then subtracted from RhoA-GST-containing reactions to correct for background glycohydrolase activity. The calibrated area was then transformed to pmol of nicotinamide produced using a standard nicotinamide curve. Kinetic values were calculated using GraphPad (GraphPad Software, San Diego, USA).

2.5 PCR Reactions

2.5.1 Site-directed mutagenesis

Variants were generated using site-directed mutagenesis with the QuikChange™ PCR method according to manufacturer’s instructions. Custom primer sets were obtained from Invitrogen (Massachusetts, USA) and the primer sequences can be found in Appendix Table A.1 and A.2. Gene products were analyzed on an agarose gel, and samples showing bands at the correct molecular weight were purified using a GeneJet PCR purification kit (ThermoFisher, Massachusetts, USA). The purified sample was then treated with DpnI (Thermo Fisher, Massachusetts, USA) to enzymatically digest any methylated DNA. The reaction was held at 37°C for 1 h before being heated to 80°C for 20 min to denature the enzyme. Plasmid was propagated in DH5α E. coli cells before being purified using a PureLink™ Quick Plasmid Miniprep kit (Invitrogen, Massachusetts, USA), and sent to the Advanced Analysis Center for sequencing
Successful reactions were then expressed in *E. coli* BL21 λDE3 cells and purified as previously described.

### 2.5.2 Deletion cloning

A series of forward primers were designed to exclude base pairs from residues 39 and 52, resulting in a truncated sequence. These deletions were accomplished using custom primer sets (Life Technologies), with the forward primers encoding for a *Nde*I cut-site at the beginning of the sequence and a single reverse primer that encoded for a *Not*I cut-site. The same reverse primer was used for each reaction, which annealed to the 3’-end of the sequence to ensure the C-terminus was unchanged. Primer sequences can be found in Appendix Table A.3.

Inserts were amplified using the respective primer sets according to manufacturer’s instructions. Gene products were analyzed using an agarose gel, and purified using a GeneJet PCR purification kit (ThermoFisher). The purified gene product was then enzymatically digested using *Nde*I and *Not*I at 37°C for 1 h. Afterwards, the reactions were heated to 65°C for 20 min to denature the enzymes. Digested gene products were then ligated into a linearized pET-28a+ vector using a molar ratio of 6:1 insert to vector. Vector digestion followed the protocol above, with the addition of 1.5 units of alkaline phosphatase (New England BioLabs) after 20 min at 37°C to prevent re-annealing. The ligation reaction was run at 16°C for 22 h using T4 DNA ligase (New England BioLabs). Plasmid was propagated and purified as described, and sent for sequencing. Successful reactions provided vectors that were then expressed in *E. coli* BL21 λDE3 cells and purified as previously described.
2.6 Growth-Deficiency Assay

*Saccharomyces cerevisiae* BY4741 were tested against LarvinA and each catalytic variant in a growth-deficiency assay previously described by Turgeon *et al.* (36). Briefly, electrocompetent cells were co-transformed with linearized pRS415-CUP1 and larvinA insert with the intention to produce pRS415-CUP1-larvinA through homologous recombination. Transformants confirmed to contain the desired vector through sequencing were then incubated overnight in SD-LEU selective media before being diluted to OD$_{595} = 2 \times 10^{-4}$. Protein expression was induced with copper sulfate concentrations ranging from 0 – 0.75 mM, after which cultures were aliquoted into a 96-well plate, sealed, and incubated at 30°C for 48 h. Absorbance measurements were then taken at 595 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany), and compared to a positive control containing the catalytic domain of the mART toxin, *Pseudomonas aeruginosa* ExoA. Each sample had 4 technical replicates and were repeated for a total of 12 biological replicates.

2.7 Macrophage Cell Entry Assay

*J774A.1* mouse macrophage cells were grown in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum in the presence of penicillin-streptomycin antibiotic (100 U/mL penicillin; 100 µg/mL streptomycin). Cells were grown in 25 cm$^2$-breathable flasks with 5% CO$_2$ and passaged at 80-90% confluency. Cells were lifted through scraping and diluted 10-fold into the medium listed above.

Confluent *J774A.1* cells were used for morphological assays to assess the effect of LarvinA on living cells. Cells were diluted to $2.5 \times 10^5$ cells/mL in the presence of 30 and 300 nM toxin
concentrations. Then, 150 μL of cell suspension was added in triplicate to a 96-well plate and allowed to incubate for 20 h. After this period of time, cells were assessed under a microscope and morphological changes were noted.

2.8 Fluorescence Microscopy

J774A.1 mouse macrophage cells were prepared as previously described. Cells were diluted to 2.5 x 10^5 cells/mL in warmed DMEM and were seeded overnight onto sterile glass coverslips. The next day, cells were treated with 300 nM of toxin-Dylight 488 conjugate and incubated for four h.

Purified protein was conjugated with Dylight 488 in 0.5 M NaCl, 0.1 M Na_3PO_4, pH 7.5 based on manufacturer’s instructions, with the exception of the molar excess of dye used. A 2-3-fold molar excess of dye was used in place of the recommended 8-15-fold due to issues with protein stability. All media was removed after four hours and cells were washed three times with PBS. Cells were then fixed with 4% PFA in PBS for 15 min on ice. Cells were washed three times with PBS and incubated with 150 mM glycine in PBS for 15 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% Triton-X-100 for 10 min at room temperature. Cells were washed with PBS before adding 2 μg/mL of DAPI in methanol to the slides. This was left to incubate for 15 min at room temperature before the final PBS wash. Note that in the case of co-localization studies, RhoA was targeted with antibodies before samples were treated with DAPI. Briefly, samples were blocked with 5% BSA in PBS for 1.5 h after permeabilization with Triton-X-100. Next, the cells were incubated with a 1/50 dilution of primary antibody (mouse monoclonal to human RhoA, Santa Cruz Biotechnology) for 1.5 h at room temperature after washing with PBS. After another round of washing with PBS, samples were incubated with a 1/1000 dilution of
secondary antibody (Alexa Fluor 594 goat anti-mouse, Invitrogen) for 1 h with mixing. Cells were washed with PBS before being treated with DAPI as previously described.

Coverslips were mounted onto glass microscopy slides using either ProLong™ Gold Antifade mountant (ThermoFisher) or DAKO Fluorescent mounting medium (Agilent Technologies). Samples were imaged through a 60 x oil immersion lens using a Nikon Eclipse Ti-S inverted microscope (Japan). Images were captured using Nikon NIS software, using an exposure time of 40 ms for DAPI and 100 ms for FITC. Images were then analyzed using ImageJ.

2.9 Assessing Metal-Binding Abilities of VTP

A gravity column containing 1 mL of Chelating FastFlow™ Sepharose was charged with 0.1 M of various divalent ions, including Ca$^{+2}$, Zn$^{+2}$ and Ni$^{+2}$. After washing the column with MQ water and equilibration buffer (50 mM Tris-HCl, 1 M NaCl, pH 7.4), 0.5 mg of protein was loaded onto the column and allowed to incubate for 30 min. The column was washed with equilibration buffer while collecting fractions. Finally, the column was washed with elution buffer (50 mM Tris-HCl, 1 M NaCl, 50 mM EDTA, pH 7.4) while collecting fractions. The fractions were analyzed using SDS-PAGE.

2.10 Statistical Analysis

Reported values are the average of triplicate measurements, and the error represents standard deviation. Statistical analysis was done in Microsoft Excel.
Chapter 3: Isolation and Characterization of LarvinA
3.1 Abstract

LarvinA and catalytic variants were purified using a combination of immobilized-metal-affinity (IMAC) and size-exclusion chromatography. The folded integrity and stability of each protein was then assessed using circular dichroism (CD) spectroscopy and thermal melt curves. Substrate-binding characterization of LarvinA found a dissociation constant of 56.2 (± 10.9) µM for the NAD$^+$ substrate, which is higher than those of C3larvin and Plx2A. However, the GH activity had comparable $K_M$ values among the three toxins, while the $k_{cat}$ of LarvinA was 4.5-fold and 200-fold larger than those of Plx2A and C3larvin, respectively. Interestingly, the transferase activity showed a biphasic curve, possibly due to a second, low affinity conformer of LarvinA. The $k_{cat}$ of this reaction was 12-fold higher than that of C3larvin but 1.6-fold lower than that of Plx2A. Finally, the cytotoxic effects of LarvinA were confirmed through cytoplasmic expression in yeast cells and exogenous treatment of macrophage cells. The catalytic variants confirmed the role of each active-site motif in enzymatic activity; however, evidence suggests that the arginine residue plays a structural role like those in the CT subgroup and not a catalytic role like other C3 toxins.

3.2 Purification and identification of LarvinA and catalytic variants

LarvinA and the catalytic variants R104A, S149A/T150A/S151A and Q187A/E189A were purified using an N-terminal poly-His$_6$-tag for IMAC in combination with size-exclusion chromatography. Purity was assessed with SDS-PAGE after each step (Fig. 3.1, Panel A). LarvinA WT gave a final yield of 0.5 mg/liter of culture, and was confirmed using MALDI-TOF mass spectrometry which showed a peak at the expected molecular weight of 27,830 Da (Fig. 3.1, Panel B). The catalytic variants R104A, S149A/T150A/S151A and Q187A/E189A yielded 4 mg/liter, 8 mg/liter and 5 mg/liter, respectively.
Figure 3.1: Purification and identification of recombinant LarvinA. (A) SDS-PAGE analysis of LarvinA purity after IMAC (Lane 2) and size-exclusion chromatography (Lane 3) compared to BioRad Precision Plus Protein Standards (Lane 1). (B) MALDI-TOF analysis of purified protein revealed a single peak at the expected molecular weight of recombinant LarvinA.

3.3 Folded integrity and thermostability of LarvinA and catalytic variants

The folded integrity and secondary structure of LarvinA was assessed using CD spectroscopy (Fig 3.2). The spectrum showed distinct secondary structure, and analysis revealed 54% of the protein was α-helical while 31% resided in β-sheets. There were no distinct differences between WT and variant spectra, indicating that the residue substitutions did not affect overall folded integrity of the variant enzymes. Next, the melting temperature ($T_m$) of each protein was measured using a StepOnePlus Real-time PCR system and SYPRO Orange™ as the indicator dye (Table 3.1). LarvinA had a $T_m$ of 62.5°C, which is higher than that of Plx2A (54.9°C) and C3larvin
The 2.7°C difference between LarvinA and C3larvin implies a contribution from the N-terminus to protein stability.

LarvinA WT had the highest $T_m$, while those of the catalytic variants were reduced as much as 2.4°C. This shows a mild reduction in thermostability, with the R104A substitution showing the largest change and the S149A/T150A/S151A substitutions showing the smallest change. These results contradict the literature on C3-exotoxins, which describe the STS motif as a structural motif while the arginine orients NAD$^+$ for catalysis (14, 37). In other C3-toxins, the STS motif contributes to active-site integrity through hydrogen bonding with residues both around and within the binding cleft. This includes the catalytic glutamate and glutamine which become properly oriented for the reaction. Conversely, the functional group of arginine forms hydrogen bonds with the phosphate groups of NAD$^+$, holding the molecule in place for hydrolysis (14, 37). Substituting this residue should, therefore, affect kinetics but have little effect on thermal stability. However, the 2.4°C shift would suggest that R104 is contributing more to protein stability than previously thought. It is worth noting that in other mART families, including the CT-group, the arginine residue contributes to stability in a similar manner as the STS motif of C3-toxins (14). Here, the arginine forms hydrogen bonds with residues surrounding the active site and stabilizes the binding cleft (14). It is therefore possible that LarvinA shares qualities with the CT-group, as well as with the C2- and C3-subgroups.
Figure 3.2: Comparison of thermostability and folding integrity of LarvinA and catalytic variants. (A) Circular dichroism spectra of LarvinA WT and catalytic variants. Each curve represents an average of nine scans. (B) Derivative fluorescent curves for LarvinA WT and catalytic variants, in which the curve minimum represents the respective T_m. Each curve represents an average of three thermograms using the SYPRO Orange™ thermal shift assay.

Table 3.1: T_m values derived from the SYPRO Orange thermal shift assay.

<table>
<thead>
<tr>
<th>Protein</th>
<th>T_m (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3larvin</td>
<td>59.8</td>
</tr>
<tr>
<td>LarvinA</td>
<td>62.5 ± 0.1</td>
</tr>
<tr>
<td>LarvinA Q188A/E190A</td>
<td>61.8 ± 0.2</td>
</tr>
<tr>
<td>LarvinA S150A/T151A/S152A</td>
<td>62.0 ± 0.2</td>
</tr>
<tr>
<td>LarvinA R105A</td>
<td>60.1 ± 0.1</td>
</tr>
</tbody>
</table>
3.4 Catalytic characterization of LarvinA

3.4.1 Affinity for NAD$^+$

The affinity for NAD$^+$ of each protein was measured using a tryptophan-fluorescence quenching assay (Fig 3.3, Table 3.2). The dissociation constant of LarvinA was determined to be 56 (± 11) µM which is higher than that of Plx2A (33 ± 2 µM) and C3larvin (21 ± 3 µM) (16, 21). While this indicates a weaker affinity for the NAD$^+$ substrate, it is still comparable to C3-toxins, including C3bot1 (60 ± 6 µM) (38). In matching with the results from the thermal shift assay, the $K_D$ of R104A (63 ± 13 µM) was comparable to that of LarvinA WT. This implies that the residue does not contribute to NAD$^+$-binding, lending more evidence to a structural role. The S149A/T150A/S151A variant shows an increased affinity for NAD$^+$, with a 1.7-fold decrease in $K_D$ (34 ± 3 µM). Interestingly, this was previously observed in Plx2A where the substitution of the STS motif with AAA caused a 1.2-fold decrease in the dissociation constant (21). It is possible that the removal of the STS motif disrupts hydrogen bonds, which the protein remakes with the NAD$^+$ substrate resulting in improved affinity. Finally, the Q187A/E189A variant shows a 2.5-fold increase in $K_D$ (143 ± 13 µM). This is inconsistent with other mART toxins which have shown no difference in binding after substituting alanine residues for the Q-X-E motif (39). These results implicate this motif in substrate binding as well as the transferase reaction.

Interestingly, none of the catalytic variants showed saturated binding at high NAD$^+$ concentrations, unlike LarvinA WT. While the first segment of the curves appears to be hyperbolic, a second, linear segment is apparent at higher ligand concentrations. This linear portion is indicative of non-specific binding which cannot be saturated, and therefore the binding curves do not reach a plateau. Disruption of these key motifs may be increasing the flexibility of the
active-site loop, allowing for multiple substrates to enter the binding cleft or opening a second binding site. These data were fit with a one site total-binding model on GraphPad.
Figure 3.3: NAD⁺ binding and hydrolysis by LarvinA. (A) NAD⁺ binding of LarvinA and catalytic variants was assessed with β-NAD⁺ substrate using a Trp quenching assay. β-NAD⁺ was titrated into the protein solution with an initial volume of 600 μL and an enzyme concentration of 1.25 μM (Error bars, S.D.). (B) Michaelis-Menten plot of LarvinA WT glycohydrolase activity against ε-NAD⁺. The reaction was held at 25°C, with LarvinA at a final concentration of 20 μM in 50 mM NaCl and 20 mM Tris, pH 7.9. The reaction was monitored for 5 minutes and repeated in triplicate (Error bars, S.D.).

Table 3.2: Binding affinity and kinetic parameters of LarvinA catalytic variants against NAD⁺.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}$ (min⁻¹) $\times 10^{-3}$</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LarvinA</td>
<td>107 ± 21</td>
<td>261 ± 20</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>LarvinA Q188A/E190A</td>
<td>~0b</td>
<td>10 ± 2</td>
<td>143 ± 13</td>
</tr>
<tr>
<td>LarvinA</td>
<td>~0b</td>
<td>5 ± 0.1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>LarvinA R105A</td>
<td>~0b</td>
<td>8 ± 0.2</td>
<td>63 ± 13</td>
</tr>
</tbody>
</table>

aEach value is the average of three replicates ± S.D. of glycohydrolase activity.

bThe $K_M$ value could not be determined accurately due to the extremely weak enzymatic activity.
3.4.2 Glycohydrolase activity

The GH activity of LarvinA was measured using a fluorescence-based assay with ε-NAD⁺ as the target substrate (Fig. 3.3, Table 3.2). The reaction showed Michaelis-Menten kinetics with a $K_M = 107 \pm 21 \, \mu M$ and a $k_{cat} = 261 \pm 20 \times 10^{-3} \, \text{min}^{-1}$. The $K_M$ is comparable to those seen in Plx2A (176 ± 8 µM) and C3larvin (120 ± 16 µM). However, the $k_{cat}$ is 4.5-fold larger than that of Plx2A (58 ± 1.0 x $10^{-3} \, \text{min}^{-1}$) and is 200-fold larger than that of C3larvin (1.3 ± 0.05 x $10^{-3} \, \text{min}^{-1}$) (16, 21). This showcases a significant improvement in catalytic efficiency with the extension of the N-terminus in LarvinA. It is likely that the additional residues are structurally supporting the active site, maintaining a more effective conformation. Additionally, the $k_{cat}$ values for R104A, S149A/T150A/S151A and Q187A/E189A were $8 \pm 0.2 \times 10^{-3} \, \text{min}^{-1}$, $5 \pm 0.1 \times 10^{-3} \, \text{min}^{-1}$, and $10 \pm 2 \times 10^{-3} \, \text{min}^{-1}$, respectively. The variants show between 27- to 49-fold decrease in turnover number, confirming the importance of these residues in the hydrolysis reaction of the NAD⁺ substrate.

3.4.3 Transferase activity

LarvinA was proven to covalently modify RhoA with ADP-ribose through a fluorescein-NAD⁺ blot (Fig 3.4). Within the substrate analogue, the fluorescein molecule is linked to the ADP-ribose moiety, and this probe will fluoresce when exposed to near UV light. The successful transfer of the ADP-ribose to a target substrate will, therefore, result in fluorescein fluorescence of that substrate. It can be seen in the SDS-PAGE gel that both the positive control reaction with Plx2A and the reaction with LarvinA produced fluorescent bands at the molecular weight of RhoA, as well as a faint band at the top of the gel. This top band is likely precipitated RhoA that did not run through the gel. In the negative control reaction, there is only a single fluorescent band at the very
bottom of the gel. This corresponds to unreacted fluorescein-NAD⁺ while the RhoA band remains nonfluorescent.

The transferase activity against NAD⁺ and RhoA was characterized using an HPLC-based assay. β-NAD⁺ was held at 300 μM while RhoA was varied, and RhoA was then held at 20 μM while β-NAD⁺ was varied. The reaction was initiated through the addition of LarvinA at a final concentration of 1 μM and allowed to continue for 5 minutes in reaction buffer (5 mM MgCl₂, 150 mM NaCl and 20 mM Tris-HCl, pH 7.4). Mobile phase (95% 20 mM phosphate buffer pH 5.5 and 5% acetonitrile) containing an internal standard was added to stop the reaction. The resulting curves were biphasic, with the first segment following Michaelis-Menten kinetics and the second appearing linear (Fig 3.5). This linear portion could be due to a second conformer in solution that has a significantly lower affinity for RhoA. The result would be a second Michaelis-Menten graph overlaid with the curve from the high affinity conformer. This second conformer may require more substrate to saturate the binding pocket, and therefore might be expected to eventually overtake the first curve, as is seen in Figure 3.6, panel B. Interestingly, this second curve did not saturate even at extremely high concentrations of substrate.

The hyperbolic segment of the curve was used to determine the kinetic parameters. A $K_M = 75 ± 10$ μM was calculated for the β-NAD⁺ substrate and for the RhoA substrate, a $K_M = 5 ± 1$ μM was calculated. This reaction had a $k_{cat} = 61 ± 3$ min⁻¹. LarvinA therefore has a lower affinity towards the β-NAD⁺ substrate, but a higher affinity for RhoA when compared to C3larvin ($K_M = 34 ± 12$ μM and $17 ± 3$ μM, respectively) (16). Unlike the GH reaction, the turnover number of LarvinA is around 12-fold higher than C3larvin ($k_{cat} = 5 ± 0.2$ min⁻¹) but 1.6-fold smaller than that of Plx2A ($k_{cat} = 99 ± 9$ min⁻¹) (16, 21).
Figure 3.4: LarvinA transfers an ADP-ribose to RhoA. LarvinA was reacted with 40 μM of RhoA-GST and 25 μM of fluorescein-NAD⁺ in 5 mM MgCl₂, 150 mM NaCl and 20 mM Tris-HCl, pH 7.4. Reactions were incubated for 1 hour at room temperature before being stopped through the addition of Laemmli buffer. BioRad Precision Plus Protein Standards (Lane 1); negative control containing only RhoA-GST and fluorescein-NAD⁺ (Lane 2); 1 μM LarvinA (Lane 3); 1 μM Plx2A (Lane 4).
Figure 3.5: Kinetic characterization of LarvinA transferase activity. Transferase activity was characterized using an HPLC-based assay with an enzyme concentration of 1 μM. Reactions were carried out in 5 mM MgCl₂, 150 mM NaCl and 20 mM Tris-HCl, pH 7.4, for 5 minutes before being stopped through the addition of mobile phase containing an internal standard. Using an Agilent Captiva filtration plate, all proteinaceous material was removed, and the remaining reaction products were separated on a reverse-phase C18 Zorbax column (Agilent technologies). The resulting nicotinamide peaks were quantified using the internal standard, 4-aminobenzoic acid. (A) Michaelis-Menten graph of LarvinA transferase activity when β-NAD⁺ was held at 300 μM and RhoA was varied. (B) Biphasic curve of LarvinA transferase activity when RhoA was held at 20 μM and β-NAD⁺ was varied.
3.5 LarvinA is toxic to *Saccharomyces cerevisiae*

LarvinA was expressed in *Saccharomyces cerevisiae* under the control of a copper-inducible promoter. A previously characterized mART toxin, ExoA, was used in the study as a positive control and an empty vector was used as a negative control. LarvinA proved to be incredibly toxic, with no growth being visible even without the addition of copper (Fig 3.5). Copper is a required cofactor for eukaryotic cell growth, and therefore is always present in small quantities. These low levels of copper are enough to cause the transcription and translation of minute quantities of protein; which, in the case of LarvinA, was enough to eliminate host cell growth. The catalytic variants were also tested in this assay. As expected, LarvinA Q187A/E189A was devoid of catalytic activity and had no effect on cell viability at any copper concentration, confirming its role in the enzymatic activity of the protein. At the highest copper concentration of 0.75 mM, the S149A/T150A/S151A variant showed an 89% decrease in culture growth while R104A completely eliminated growth. This contributes to the evidence suggesting that R104 behaves as a structural residue, since its removal did not improve cell viability.

3.6 Cell entry against mouse macrophage cells

The mouse macrophage cell line J774A.1 was treated with nanomolar concentrations of LarvinA. Unlike C3larvin, both 30 nM and 300 nM treatments elicited morphology changes within the cells (Fig 3.7). These changes represent a loss of regulation within the actin cytoskeleton, and often appear as thin protrusions from a rounded cell (40). This finding confirms the importance of the N-terminus in cell entry and membrane translocation. Similar to the yeast growth deficiency assay, cells treated with the Q-X-E variant showed no change in morphology, and the S-T-S and R variants caused less extreme morphology changes in only a portion of the cells.
Figure 3.6: Growth deficiency assay in *Saccharomyces cerevisiae*. LarvinA and the three catalytic variants were expressed in yeast cells under the copper-inducible promoter, CUP1. All growth was compared to that of yeast expressing a positive control toxin, *P. aeruginosa* exotoxin A catalytic domain, PE24). The negative control contained the empty plasmid, pRS414 CUP1.
Figure 3.7: LarvinA translocates the target cell membrane. The mouse macrophage cell line, J774A.1, was treated with 300 nM of toxin and incubated for 20 hours at a cell density of 2.5 x 10^5 cells/mL. Infected cells appear elongated due to cytoskeletal dysregulation resulting from the inactivation of RhoA. (A) Untreated cells; (B) buffer treated cells; (C) 300 nM LarvinA (D) 300 nM LarvinA Q188A/E190A; (E) 300 nM LarvinA R105A; (F) 300 nM LarvinA S150A/T151A/S152A.
3.7 Conclusions

LarvinA was discovered in *Paenibacillus larvae* ERIC III using *in silico* genome sequencing. It is nearly identical to the previously characterized C3larvin, except for an additional 58 residues at the N-terminus and a single residue substitution at position 2 of C3larvin. Like C3larvin, LarvinA shows GH activity and targets RhoA in transferase activity; however, the kinetic parameters of each reaction differ. LarvinA is cytotoxic to yeast cells, and catalytic variants confirm that this toxicity is due to enzymatic function. Moreover, LarvinA can successfully translocate the target cell membrane where C3larvin could not. These findings implicate the N-terminus in both the catalytic and translocation functions of C3-toxins. As such, the N-terminal residue differences between C3larvin and LarvinA is the focus of the next chapter.
Chapter 4: Structural and Functional Role of the LarvaN A N-terminus
4.1 Abstract

Deletions and substitutions were made to the N-terminus of LarvinA to assess the overall contribution of helix 1 to protein structure and function. The folded integrity and stability of each protein was assessed using circular dichroism (CD) spectroscopy and thermal analysis. Interestingly, the removal of the first 20 residues from the N-terminus had little effect on protein stability; however, removing the next 13 residues caused a 2.2 – 4.3°C decrease in the protein melting temperature, T_m. The catalytic characterization of these variants revealed two residues of interest: Asp23 and Ala31. The removal of Asp23 caused a 2-fold increase in the NAD^+ substrate-binding affinity, K_D, while the deletion or substitution of Ala31 resulted in a loss of GH enzyme activity. Lastly, fluorescence microscopy experiments revealed that all tested proteins, including C3larvin (a related toxin that contains a truncated helix 1 region), were able to penetrate the target cell membrane. LarvinA WT and the N-terminal deletions showed a dispersed phenotype within the cell, whereas C3larvin appeared as large puncta. Localization studies indicated that C3larvin failed to target cellular RhoA, suggesting that C3larvin is retained in endosomes and cannot translocate into the cytoplasm.

4.2 Cloning and purification of N-terminal variants

A series of deletions were made to the N-terminus of LarvinA. It was hypothesized that residues of interest would be found before the start of the naturally truncated C3larvin sequence but shared between LarvinA and Plx2A, since Plx2A was shown to also target macrophage cells. The first set of deletions, therefore, produced a final protein equal in size to either (1) C3larvin or (2) Plx2A, resulting in LarvinA ΔY2-W34 and LarvinA ΔY2-A21, respectively. Other regions were specifically targeted based on a multiple-sequence alignment performed by Dr. Miguel Lugo (PDF
in the Merrill lab) (Fig. 4.1). This alignment compared the N-terminus of both C2- and C3-like mART toxins and highlighted multiple groups of shared residues. Specifically, the shared cluster between all toxins is depicted in purple, and the cluster shared between C2 and C2/C3 toxins is depicted in yellow. These residues were also targeted for site-directed mutagenesis experiments in the creation of LarvinA F24A/A31L/W34A and D23A/K25A/D27A/R28A variants. The purification of all variants followed the protocol previously outlined, and proteins yields can be found in Table 4.1.
Figure 4.1: Multiple-sequence alignment of the N-terminus of C3-, C3/C2-, and C2-like toxins. Residues conserved in all groups are shown in purple; residues conserved in C3- and C3/C2-like toxins are shown in pink; residues conserved in C3/C2- and C2-like toxins are shown in yellow; residues conserved in C3-like toxins are shown in gray. Italicized residues denote deviations from otherwise conserved positions. Gaps within the sequence are denoted by (.) and continuation within a sequence is denoted by (-). Modified from Miguel Lugo (41).

Table 4.1: Protein yields of LarvinA N-terminal variants.

<table>
<thead>
<tr>
<th>LarvinA Variant</th>
<th>Protein yield (mg) per litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔY2-A21</td>
<td>0.5</td>
</tr>
<tr>
<td>ΔY2-D23</td>
<td>1</td>
</tr>
<tr>
<td>ΔY2-K25</td>
<td>1</td>
</tr>
<tr>
<td>ΔY2-D27</td>
<td>1</td>
</tr>
<tr>
<td>ΔY2-A30</td>
<td>1</td>
</tr>
<tr>
<td>ΔY2-K33</td>
<td>4</td>
</tr>
<tr>
<td>ΔY2-W34</td>
<td>10</td>
</tr>
<tr>
<td>F24A/A31L/W34A</td>
<td>4</td>
</tr>
<tr>
<td>D23A/K25A/D27A/R28A</td>
<td>1</td>
</tr>
</tbody>
</table>
4.3 Folded integrity and stability of N-terminal variants

The folded integrity of each variant was assessed using CD spectroscopy. The resulting curves show nearly identical secondary structure patterns compared to WT; however, the graphs are shifted towards the 0 molar ellipticity value on the y-axis. This was expected since the percentage of residues lying in α-helices and random coils is being reduced with each deletion, while the percentage of residues found in β-sheets remains the same. Next, the thermal stability of each protein was measured using the previously described system (Table 4.2). The $T_m$ values of each variant were up to 4.5°C lower than LarvinA WT. Interestingly, the smallest change in $T_m$ values was seen in LarvinA ΔY2-A21 ($T_m = 62.4°C$). This value is equivalent to that of LarvinA WT (62.5°C), validating that residues in the initial N-terminal region that are not shared between LarvinA and Plx2A do not contribute significantly to structural stability in LarvinA. The greatest shift was seen in LarvinA D23A/K25A/D27A/R28A with a melting temperature of 58.0°C. Replacing these charged residues with alanine likely impaired hydrogen bonding within the N-terminus, reducing structural stability of the enzyme. Of note, the ΔY2-W34 deletion had a $T_m = 58.7°C$, while C3larvin had a $T_m = 59.6°C$. The only difference between the two proteins is a single residue; where LarvinA has a lysine, C3larvin has a glutamate residue. This change in charge is expected to be mildly destabilizing, explaining the observed shift in $T_m$. 
Table 4.2: $T_m$ values of LarvinA N-terminal variants derived from the SYPRO Orange thermal shift assay.

<table>
<thead>
<tr>
<th>LarvinA Variant</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔY2-A21</td>
<td>62.4 ± 0.2</td>
</tr>
<tr>
<td>ΔY2-D23</td>
<td>58.6 ± 0.5</td>
</tr>
<tr>
<td>ΔY2-K25</td>
<td>57.3 ± 0.1</td>
</tr>
<tr>
<td>ΔY2-D27</td>
<td>59.4 ± 0.4</td>
</tr>
<tr>
<td>ΔY2-A30</td>
<td>60.0 ± 0.3</td>
</tr>
<tr>
<td>ΔY2-K33</td>
<td>59.3 ± 0.1</td>
</tr>
<tr>
<td>ΔY2-W34</td>
<td>58.6 ± 0.2</td>
</tr>
<tr>
<td>F24A/A31L/W34A</td>
<td>58.4 ± 0.3</td>
</tr>
<tr>
<td>D23A/K25A/D27A/R28A</td>
<td>57.9 ± 0.1</td>
</tr>
</tbody>
</table>

Figure 4.2: Circular dichroism spectra of LarvinA N-terminal variants. (A) Spectra of the N-terminal deletions. Each curve represents the average of nine readings. (B) Spectra of N-terminal variants. Each curve represents the average of nine readings.
4.4 Catalytic characterization of N-terminal variants

4.4.1 Affinity for NAD$^+$

The affinity of each variant for the NAD$^+$ substrate was measured using the previously described tryptophan-quenching assay (Table 4.3). The only variant that showed a $K_D$ value equal to LarvinA WT was the ΔY2-A21 deletion ($K_D = 59 \pm 2 \mu M$). This indicates that the first 20 residues do not contribute significantly to substrate binding. The largest change in dissociation constant was seen in LarvinA D23A/K25A/D27A/R28A and LarvinA ΔY2-D23 with $K_D$ values of $140 \pm 14 \mu M$ and $117 \pm 10 \mu M$, respectively. This implicates residue Asp23 in NAD$^+$ binding, although removing Lys25, Asp27 and Arg28 further reduces $K_D$. Interestingly, as more residues are removed from the N-terminus, the dissociation constant becomes smaller, indicating an increase in NAD$^+$ substrate-binding affinity. This may be indicative of a conformational change within the active-site loop, resulting from altered residue packing within the truncated N-terminus.

When comparing the crystal structure of C3larvin to other C3-toxins, the ARTT loop, or active-site, shows some conformational differences (Fig. 4.3). Other C3-toxins show a distinct fold within the ARTT loop, whereas C3larvin appears to have a more open conformation. This increased flexibility introduced into the active-site loop could be contributing to the lower $K_D$ values seen in the larger N-terminal deletions. The largest deletion, LarvinA ΔY2-W34, had a $K_D = 38\pm 2 \mu M$ which is similar to C3larvin ($K_D = 21 \pm 3 \mu M$).
Table 4.3: Binding affinity and kinetic parameters of LarvinA N-terminal variants against NAD⁺.

<table>
<thead>
<tr>
<th>LarvinA Variant</th>
<th>K_M (μM)</th>
<th>k_cat (min⁻¹) x 10⁻³</th>
<th>k_cat/K_M x 10⁻⁵</th>
<th>K_D (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔY2-A21</td>
<td>120 ± 31</td>
<td>100 ± 10</td>
<td>83</td>
<td>59 ± 2</td>
</tr>
<tr>
<td>ΔY2-D23</td>
<td>160 ± 23</td>
<td>46 ± 4</td>
<td>31</td>
<td>117 ± 10</td>
</tr>
<tr>
<td>ΔY2-K25</td>
<td>104 ± 27</td>
<td>19 ± 2</td>
<td>18</td>
<td>102 ± 3</td>
</tr>
<tr>
<td>ΔY2-D27</td>
<td>112 ± 17</td>
<td>12 ± 0.7</td>
<td>11</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>ΔY2-A30</td>
<td>84 ± 17</td>
<td>7 ± 0.4</td>
<td>8</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>ΔY2-K33</td>
<td>NtDt</td>
<td>NtDt</td>
<td>NtDt</td>
<td>75 ± 7</td>
</tr>
<tr>
<td>ΔY2-W34</td>
<td>NtDt</td>
<td>NtDt</td>
<td>NtDt</td>
<td>38 ± 9</td>
</tr>
<tr>
<td>F24A/A31L/W34A</td>
<td>NtDt</td>
<td>NtDt</td>
<td>NtDt</td>
<td>85 ± 14</td>
</tr>
<tr>
<td>D23A/K25A/D27A/R28A</td>
<td>28 ± 6</td>
<td>46 ± 2</td>
<td>166</td>
<td>140 ± 14</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates ± S.D. NtDt = not detectable.

Figure 4.3: Comparison between the active-site loops of C3larvin and other C3-toxins. All toxins are depicted as cartoons. C3larvin (PDB ID: 4TR5, coloured light blue, active site coloured purple) was structurally aligned with C3bot1 (PDB ID: 2C8B, coloured gray80, active site coloured density), C3lim (PDB ID: 3BW8, coloured gray40, active site coloured blue) and Plx2A (PDB ID: 5URP, coloured gray70, active site coloured tv_blue) using PyMOL ver 1.3
4.4.2 Glycohydrolase activity

Each variant was tested against the ε-NAD$^+$ substrate to assess GH activity (Table 4.3). All variants showed similar $K_M$ values to that of WT indicating that the ability of these modified enzymes to interact with the NAD$^+$ substrate is relatively unchanged—except for D23A/K25A/D27A/R28A. The altered $K_M$ is likely a result of hydrogen bond rearrangement within the protein. To compensate for lost interactions induced by deletion of N-terminal residues, new hydrogen bonds and other non-covalent interactions may be established with the NAD$^+$ substrate within the active site, leading to an increased affinity for the substrate. This was also reflected in the specificity constant of D23A/K25A/D27A/R28A, which is greater than all other N-terminal variants indicating that it is a more efficient enzyme by comparison. When considering the enzymatic activity between the variant proteins, there is a clear decrease in $k_{cat}$ with each deletion. Removal of the first 20 residues decreased the $k_{cat}$ by 2.6-fold, but removing just two more residues further reduces the $k_{cat}$ by another 2.3-fold (Fig. 4.4). This pattern continues until the reaction is undetectable. It can be safely assumed that the loss of Asp23, and not Lys25, is responsible for the decrease in $k_{cat}$ due to the same value being reported for D23A/K25A/D27A/R28A. Interestingly, the enzyme reaction is undetectable past the removal of residue Lys33 or in the F24A/A31L/W34A variant. These results imply that the replacement of Ala31 is responsible for the loss in activity.
4.5 Cell entry against mouse macrophage cells

4.5.1 Morphology assay

All variants were tested against J774A.1 mouse macrophage cells at a final concentration of 300 nM. There is an inverse relationship between N-terminus length and the extent of morphology changes (Fig. 4.5). As stated previously, morphology changes represent a loss of regulation in the actin cytoskeleton causing the formation of thin protrusions from the cell. The larger deletions caused visibly smaller changes to the cell when compared to WT. However, it is unclear whether this correlation is due to the toxins ability to penetrate the cell membrane or due to weakened catalytic activity. Since this assay could not differentiate between cell entry and toxin activity within the cell, fluorescence microscopy experiments were developed.
Figure 4.5: LarvinA N-terminal deletions have varying effects on the morphology of J774A.1 macrophage cells. The mouse macrophage cell line was treated with 300 nM of toxin and incubated for 20 hours at a cell density of $2.5 \times 10^5$ cells/mL. The larger the deletion, the smaller the effects on macrophage morphology. Buffer control (A); LarvinA WT (B); LarvinA ΔY2-A21 (C); LarvinA ΔY2-D23 (D); LarvinA ΔY2-K25 (E); LarvinA ΔY2-D27 (F); LarvinA ΔY2-A30 (G); LarvinA ΔY2-K33 (H); LarvinA ΔY2-W34 (I).
4.5.2 Fluorescence microscopy

Purified protein was conjugated to Dylight 488, allowing the protein to be visualized with a green fluorophore. Cells were then incubated with 300 nM of conjugated protein for 4 h before being washed, fixed and treated with DAPI to stain the nucleus. LarvinA WT, LarvinA N-terminal variants and C3larvin were tested against the macrophages. Interestingly, C3larvin was visible in this assay and appeared as clustered puncta within the cell (Fig. 4.6). This phenotype was distinctly different from that of LarvinA and its N-terminal variants. Where C3larvin seemed to form a single, large cluster within an individual cell, LarvinA appeared dispersed in smaller patches. Interestingly, the total N-terminal deletion, ΔY2-W34, behaved in a similar manner to LarvinA WT. This suggests that the N-terminus may not be specifically responsible for cell entry. Attention was then shifted towards the single residue difference between LarvinA ΔY2-W34 and C3larvin. The variant LarvinA ΔY2-W34 K2E was then introduced into the assay, and it showed a similar phenotype to C3larvin. This suggests that Lys36 in LarvinA plays an important role in the translocation activity of the *P. larvae* C3 toxins.
Figure 4.6: Fluorescence microscopy of J774A.1 mouse macrophage cells treated with toxins conjugated with Dylight 488. Macrophage cells were seeded overnight at $2.5 \times 10^5$ cells/mL before being treated with 300 nM of toxin for 4 h. Samples were exposed for 40 ms at 358 nm for DAPI and 100 ms at 495 nm for FITC. Data was analyzed using ImageJ. LarvinA WT (A); LarvinA ΔY2-A31 (B); LarvinA ΔY2-W34 (C); C3larvin (D); LarvinA ΔY2-W34 K2E (E).
4.5.3 Co-localization with RhoA

Before further investigating the residue substitution between LarvinA ΔY2-W34 and C3larvin, it was important to better understand the different phenotypes seen in C3larvin- and LarvinA-treated cells. The variations seen under fluorescence microscopy likely indicated that the two proteins were not localizing to the same cellular compartments. Given that C3larvin failed to cause morphology changes within the cells, it is reasonable to assume that the protein was incapable of targeting, or acting on RhoA in live cells, despite its ability to modify Asn41 on RhoA in vitro (16). Conversely, LarvinA caused extensive morphology changes indicating that it was targeting the G-like protein. Co-localization experiments, therefore, targeted cellular RhoA using antibodies conjugated to Alexa Fluor 595 which contains a red fluorophore. As expected, the green fluorescence from LarvinA overlaid with the red fluorescence from RhoA, giving an overall yellow colour (Fig. 4.7). In C3larvin-treated samples, however, the red fluorescence associated with RhoA is much more apparent and does not overlay well with the green fluorescence imparted by the toxin-conjugate. The failure of C3larvin to target cellular RhoA, and the appearance of large clustered patches of the toxin within the cell, could be an indication that C3larvin enters the cell through specific internalization; however, it cannot escape the endosome once inside the host macrophage.

The pathway taken by C3-toxins once inside the cell is unknown; however, it has been characterized for Cholera toxin (CT). CT travels to the endoplasmic reticulum (ER) of the cell through retrograde trafficking, where the catalytic subunit then unfolds (42). It escapes the ER using the degradation pathway meant to target misfolded cellular proteins. Once in the cytoplasm, CT refolds and targets Gs for ADP-ribosylation (42). It is possible that C3-toxins follow similar
steps, in that they are internalized and must travel through various cellular compartments before moving into the cytoplasm to target RhoA. If this is the case, then C3larvin could be getting trapped in vesicles at some point during the pathway which is why it fails to intoxicate target cells. This theory is currently being investigated.

Figure 4.7: Co-localization of LarvinA and C3larvin with cellular RhoA. Macrophage cells were seeded overnight at 2.5 x 10^5 cells/mL before being treated with 300 nM of toxin conjugated with DyLight 488 for 4 hrs. Samples were exposed for 40 ms at 358 nm for DAPI, 100 ms at 495 nm for FITC, and 100 ms at 532 nm for TRITC. Data was analyzed using ImageJ. LarvinA treated cells (A); C3larvin treated cells (B).
4.6 Conclusions

Deletions and substitutions were made to the N-terminus of LarvinA, resulting in decreased protein stability and catalytic efficiency. The catalytic characterization of these variants identified two residues of interest: Asp23 and Ala31. Removal of Asp23 caused a 2-fold increase in the $K_D$ for NAD$^+$ substrate binding, indicating a loss of affinity for the NAD$^+$ substrate. Alternatively, each N-terminal deletion resulted in a decrease in $k_{cat}$; however, it was not until the removal of Ala31 that the reaction was undetectable. These effects were also noted in the D23A/K25A/D27A/R28A and F24A/A31L/W34A variants, respectively. Single-residue substitutions of Asp23 and Ala31 were further investigated and are discussed in the next chapter.

Each variant was tested against mouse macrophage cells to assess the effect on cell morphology. The extent of the morphology changes varied based on the size of the deletion. From the kinetic data, it was clear that the morphology assay would not be enough to indicate membrane translocation. The fluorescence microscopy experiments were, therefore, developed and showed that even the largest deletion was able to enter the cell. However, this phenotype was different from the one seen in C3larvin-treated cells. Furthermore, while LarvinA appears to localize with RhoA, C3larvin does not. C3larvin may be internalized in the host macrophages but cannot traverse into the cytoplasm to target intracellular RhoA. This will require further investigation into the cellular pathway taken by C3 toxins. Additionally, these findings brought attention to the single residue difference between C3larvin and LarvinA. The role of Lys36 is also discussed in the next chapter.
Chapter 5: Investigation into active-site supportive residues: Asp23, Ala31 and Lys36
5.1 Abstract

The three residues Asp23, Ala31 and Lys36 were suspected of contributing to the enzymatic or biological function of LarvinA. Analysis of the LarvinA homology model showed all three were close to catalytic motifs, and therefore may structurally support key residues. Moreover, Lys36 was found to interact with the previously described Ile153 and Tyr178 residues, which were predicted to support the orientation of helix 1 against the ARTT-loop. Variants of each of the five residues were shown to have decreased enzymatic capacity, with LarvinA D23A showing the lowest affinity for the NAD$^+$ substrate, and LarvinA A39L having the largest decrease in $k_{cat}$ value. Both Ile153 and Tyr178 were proven to be structurally supportive residues in the active-site as predicted, since substituting either residue caused a 5 or 4°C decrease in $T_m$, respectively, while also decreasing the rate of catalysis by 7- or 33-fold, respectively. Lys36 was also proven to be enzymatically significant, since substitution of this residue caused up to a 4-fold decrease in $k_{cat}$. However, this residue was also proven to be globally destabilizing, since replacing this lysine caused an increase in thermal stability. Finally, fluorescence microscopy experiments were carried out to elucidate the role of Lys36 in cell toxicity. All full-length toxins appeared dispersed with the cytoplasm, regardless of the residue at position 36. Alternatively, reversing the charge at the corresponding position in N-terminally truncated proteins caused the toxin to appear as large puncta within the cell. This difference may be explained by the change in protein dipole and, therefore, pH-dependency as previously described through computational studies (41); however, this will require further investigation.
5.2 Analysis of the LarvinA homology model

The three residues of interest are conserved between either the C2- or C3-subgroups in mART toxins, suggesting that they likely contribute to toxin function. The LarvinA homology model was analyzed to assess residue orientation and possible interactions (Fig. 5.1). Both Asp23 and Ala31 were found to be in close contact (within 4 Å) with residues surrounding key catalytic motifs. Specifically, Asp23 was in contact with Gln102 and Tyr104, which surround the catalytic Arg105 residue (Fig. 5.2). Based on results previously described, Arg105 does not contribute to substrate binding and, therefore, removing residues supporting it should not impact the $K_D$ value for NAD$^+$ substrate binding. However, further analysis showed that Gln102 and Tyr104, while close in sequence to Arg105, are structurally near Thr150 and Ser151 of the S-T-S motif. Interactions between this series of residues are likely responsible for proper orientation of the S-T-S motif for NAD$^+$ substrate binding and catalysis.

Both the deletion and substitution of Ala31 produced largely inactive variants. Alanine residues rarely contribute to catalysis; however, they can be important for active-site motif orientation and residue packing. Further analysis of the LarvinA model shows that Ala31 faces towards the center of the protein where it is close to Ile153 and Val154, which is immediately after the S-T-S motif (Fig 5.2). Again, while these residues are close in sequence to the S-T-S motif, they are also structurally close to Tyr189 which sits in-between the Q-X-E motif. The bulky, aromatic side-chain of tyrosine likely needs to be oriented away from the catalytic center. Consequently, the active-site residues would be oriented towards the substrate-binding pocket due to steric hindrance. Based on the structure and the catalytic data, Ala31 appears to play a structural role to help orient
the Q-X-E motif and possibly the S-T-S motif. The alanine residue is totally conserved between both C3- and C2-toxins suggesting it may play a critical role in both toxin subgroups.

Lys36 was identified as a possible contributor to membrane translocation, rather than contributing to enzymatic function as seen with the previous two residues. A pairwise sequence alignment between LarvinA and C3larvin showed a single residue substitution at position 36 of LarvinA. Simply, where LarvinA contains a lysine, C3larvin possesses a glutamate at the corresponding position. This led to the creation of LarvinA Lys36Q, R and E variants to assess a possible link between charge and membrane translocation. Interestingly, the lysine residue is conserved within LarvinA, Plx2A and other C2-toxins, suggesting it serves a functional purpose. Analysis of the LarvinA homology model revealed that Lys36 is in contact with Tyr178, Asp180, and Tyr189 (Fig. 5.2). The proximity to Tyr189, part of the Q-X-E motif, suggests a possible structural role of Lys36. Additionally, Tyr178 was previously identified through computational studies as a key structural residue that contributes the orientation of helix 1 against the ARTT-loop (41).

Tyr178 is a highly conserved residue between C2- and C3-toxins (41). Found on the β5-strand, this residue is predicted to interact with the α1-helix through hydrogen bonding and hydrophobic interactions. This series of interactions is proposed to occur between the β5-strand tyrosine, a conserved isoleucine/leucine from the substrate-binding loop, a glycine residue in helix 1 predicted to orient the β5-strand tyrosine, as well as the S-motif’ (the conserved F-A-W residues in helix 1 described in the previous chapter) (Fig. 5.2) (41). This may also help to explain the importance of Ala31. These contacts appear to be conserved in LarvinA, but interestingly, the study did not
identify a lysine residue as being structurally significant. Lys36 and Asp180 appear to form a salt-bridge which is oriented towards the phenol ring of Tyr189. This orientation, combined with the position of Tyr178, may be coordinating Tyr189 through repulsion electrostatics. To elucidate the role of these residues, LarvinA K36A, I153A and Y178A were investigated. Note that LarvinA G35L was prepared; however, the protein proved to be unstable and was excluded from the study.

Figure 5.1: Location of Asp23, Ala31 and Lys36 residues in the LarvinA homology model. LarvinA homology model is shown in cartoon with helices in lightblue, β-strands in palegreen and loops in bluewhite. Residues of interest shown as yellow spheres. Using the structural alignment tool in PyMOLv1.3, the NAD$^+$ substrate (magenta) was modelled into the active site of LarvinA using the C3bot1-NAD$^+$ complex (PDB ID: IGFZ.A) as a template.
Figure 5.2: Structural interactions proposed to orient the catalytic motifs. LarvinA homology model is shown in cartoon and set to 40% transparency with helices in lightblue, β-strands in palegreen and loops in bluewhite. All residues are shown as spheres and coloured by element. Asp23, Ala31 and Lys36 is shown in yellow, Tyr178 is shown in green, Gly35 is shown in gray, Tyr189 is shown in magenta, Ile153 is shown in cyan, and the S-motif is shown in purple. Interactions between Asp23 and the S-T-S motif (A); Interactions between Ala31 and the Q-X-E motif (B); Residue-packing of Gly35 resulting in the orientation of Tyr178 (C); The orientation of the hydroxyl group of Tyr178 and amino group of Lys36 towards the phenol ring of Tyr189 (D); Residue-packing of Ile153, Tyr178 and Tyr189 (E); Residue-packing of Ile153 against the S-motif (F).
5.3 Folded integrity and thermal stability of LarvinA single-residue variants

Folded integrity was assessed using CD spectroscopy. The resulting spectra resembled that of LarvinA WT, indicating that the variants were properly folded (data not shown). Both LarvinA D23A and A31L have reduced thermal stability, consistent with the $T_m$ values of both the N-terminal deletions and substitutions (Table 5.1). LarvinA I153A resulted in a 5°C decrease in $T_m$, confirming the role of Ile153 as a stabilizing residue (41). Found in the substrate-binding loop, this isoleucine is close to both the S-motif and Tyr178, creating a connection between the active-site and $\alpha_1$-helix. Similarly, LarvinA Y178A showed a 4°C decrease in $T_m$, again confirming the stabilizing role of these residues and their contacts (41). Conversely, substitution of Lys36 with either an arginine or glutamate residue stabilized the protein. The 3.6°C difference between LarvinA WT and LarvinA K36E suggests that a negative charge stabilizes the protein fold better than a positive or neutral charge.

Table 5.1: $T_m$ values derived from the SYPRO Orange thermal shift assay.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$T_m$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LarvinA</td>
<td>62.5 ± 0.1</td>
</tr>
<tr>
<td>LarvinA D23A</td>
<td>59.4 ± 0.1</td>
</tr>
<tr>
<td>LarvinA A31L</td>
<td>58.4 ± 0.1</td>
</tr>
<tr>
<td>LarvinA K36Q</td>
<td>61.5 ± 0.2</td>
</tr>
<tr>
<td>LarvinA K36R</td>
<td>63.2 ± 0.1</td>
</tr>
<tr>
<td>LarvinA K36E</td>
<td>66.1 ± 0.7</td>
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<tr>
<td>LarvinA K36A</td>
<td>62.8 ± 0.1</td>
</tr>
<tr>
<td>LarvinA I153A</td>
<td>57.3 ± 0.1</td>
</tr>
<tr>
<td>LarvinA Y178A</td>
<td>58.4 ± 0.1</td>
</tr>
</tbody>
</table>
5.4 Catalytic characterization

5.4.1 Affinity for NAD$^+$

The $K_D$ of each variant was measured as previously described (Table 5.3). As predicted, the substitution of Asp23 reduced the affinity for the NAD$^+$-substrate to a similar extent as the D23A/K25A/D27A/R28A and ΔY2-D23 variants ($K_D = 140$ and $117$ μM, respectively). This confirms that Asp23 contributes to NAD$^+$-binding, likely through the structural support of key residues described above. The largest increase in affinity was seen in the K36E variant. This increased affinity for the substrate, coupled with the increased thermal stability of the protein, indicates that a negative charge at this position is more favourable to protein structure and substrate-binding. It is possible that this substitution causes a conformational shift between helix I and the active-site loop, improving residue packing and affinity for the substrate. The K36A substitution also reduced $K_D$, although not to the same extent as the glutamate substitution.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}$ (min$^{-1}$) x $10^3$</th>
<th>$k_{cat}/K_M$ x $10^5$</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LarvinA</td>
<td>107 ± 21</td>
<td>261 ± 20</td>
<td>244</td>
<td>56 ± 11</td>
</tr>
<tr>
<td>LarvinA D23A</td>
<td>94 ± 28</td>
<td>7 ± 0.5</td>
<td>7</td>
<td>131 ± 27</td>
</tr>
<tr>
<td>LarvinA A31L</td>
<td>~ 0</td>
<td>~ 0</td>
<td>~ 0</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>LarvinA K36Q</td>
<td>85 ± 19</td>
<td>75 ± 6</td>
<td>88</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>LarvinA K36R</td>
<td>80 ± 15</td>
<td>63 ± 4</td>
<td>78</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>LarvinA K36E</td>
<td>72 ± 12</td>
<td>82 ± 5</td>
<td>114</td>
<td>38 ± 0.4</td>
</tr>
<tr>
<td>LarvinA K36A</td>
<td>75 ± 18</td>
<td>58 ± 4</td>
<td>76</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>LarvinA I153A</td>
<td>95 ± 13</td>
<td>40 ± 2</td>
<td>42</td>
<td>85 ± 8</td>
</tr>
<tr>
<td>LarvinA Y178A</td>
<td>99 ± 12</td>
<td>8 ± 0.3</td>
<td>8</td>
<td>65 ± 14</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates ± S.D.
5.4.2 Glycohydrolase activity

The GH activity was characterized as described in Chapter 2. Again, the A31L variant behaved as predicted, with no measurable GH activity (Table 5.2). The larger leucine side chain is most likely affecting residue-packing at this site, disrupting the series of interactions that contribute to the orientation of the Q-X-E motif. The two other residues predicted to be a part of that interaction, I153 and Y178, showed a 6- and 31-fold decrease in specificity constant when substituted with alanine, respectively. This suggests that Y178 is more structurally supportive to enzymatic function than I153. LarvinA D23A also had a 35-fold reduction in specificity constant, possibly due to its proximity to both the S-T-S motif and catalytic arginine.

The specificity constants for LarvinA K36Q, K36R and K36A were reduced by 3-fold, while LarvinA K36E was reduced by 2-fold. Despite showing increased thermal stability, the K36E substitution caused a significant decrease in enzymatic activity, albeit to a lesser extent than the other substitutions. Regardless, the lysine residue is, therefore, more favourable at this position for catalytic function. The $k_{\text{cat}}$ values of each variant were decreased as much as 4-fold compared to WT. The similarly charged K36R and neutral K36A had the lowest $k_{\text{cat}}$ values, possibly illustrating the importance of size or flexibility at this site over charge. The reduced catalytic rate suggests that Lys36 assists in the structural support of the active site, and its loss results in a conformational shift that is not conducive to enzymatic function.
5.5 Fluorescence Microscopy of LarvinA K36 Variants

The initial purpose of this study was to elucidate the role of charge at position 36 in LarvinA membrane translocation. The variants, LarvinA K36E, K36Q and K36R were all tested against macrophage cells, as previously described. Unexpectedly, all proteins showed similar phenotypes to that of LarvinA, where they appear dispersed within the cytoplasm (Figure 5.3). These results suggest that the charge at this position has no effect on membrane translocation with respect to full-length toxin function; however, the same cannot be said for the truncated toxin. As seen in the previous chapter, LarvinA WT and LarvinA ΔY2-W34 share a similar effect with respect to cellular phenotype, while C3larvin and LarvinA ΔY2-W34 K2E display a different phenotypic effect. Consequently, it appears that truncation of helix 1, coupled with the charge reversal at position 2 (in the truncated protein) is responsible for the phenotypic differences seen in fluorescence microscopy experiments.

Recently, a computational study of C2-, C3- and C2/C3-like toxins identified an N-terminal motif exclusive to the C3-toxin family (41). The cluster, E-TN-(E/D)-E-K, where the hyphens represent any number of residues, was described as a possible translocation motif based on its compatibility with previously reported data (41). Firstly, it was located in the N-terminus, shown to contribute to membrane translocation (16). As previously reported, C3larvin had no visible effect on target cell morphology until a 19-residue, N-terminal addition was made from C3bot1 (16, 41). This addition, which consisted of residues Tyr2-Trp19 from C3bot1, included the above motif. Additionally, C3bot1 and C3lim display increased toxicity under acidic conditions, and may translocate from acidified endosomes into the cytosol (40). This implies the importance of charge in this N-terminal segment, which the afore-mentioned motif may contribute. Contrary to the
above evidence, LarvinA and Plx2A do not contain this cluster of residues, yet both toxins are able to infect macrophage cells (21).

The significance of these residues may not be in their ability to form specific interactions, but could, instead, be their contribution to a global effect. Computational predictions revealed that the addition to the N-terminus of C3larvin resulted in a shift in the protein dipole and pH-dependency (41). This effect may arise from a combination of residues, rather than any single residue. This may explain how LarvinA and Plx2A can enter macrophage cells despite not sharing the above motif. It may also explain how exchanging a charge on LarvinA has little effect on cell entry and localization, as seen with LarvinA WT, K36E, K36Q and K36R, but has a large effect on a truncated protein; as seen with C3larvin and LarvinA ΔY2-W34 K2E. Analysis of the pathway taken by Cholera toxin during cell intoxication reveals multiple effector proteins used to eventually escape the vesicle system and target Gs. If C3-toxins use similar means to reach the RhoA substrate, changing the protein dipole could impact the ability of the toxin to form protein-protein interactions. It is unclear at this time whether the protein dipole is significant to membrane translocation and will require further examination.
Figure 5.3: Fluorescence microscopy of J774A.1 mouse macrophage cells treated with LarvinA K36 variants conjugated with Dylight 488. Macrophage cells were seeded overnight at 2.5 x 10^5 cells/mL before being treated with 300 nM of toxin for 4 hrs. Samples were exposed for 40 ms at 358 nm for DAPI and 100 ms at 495 nm for FITC. Data was analyzed using ImageJ. LarvinA K36Q (A); LarvinA K36E (B); LarvinA K36R (C).

5.6 Conclusions

Single-residue variants of LarvinA were investigated to elucidate novel structural interactions. Substitution of Asp23 or Ala31 negatively impacted both substrate-binding and GH activity. Analysis of the LarvinA homology model revealed that both residues are likely participating in a series of interactions resulting in the orientation of the Arg, S-T-S and Q-X-E motifs. Similarly, Lys36 contributes to the stabilization of the α1-helix against the ARTT-loop, along with previously
identified Ile153 and Tyr178 residues. As a result, Tyr189 is oriented away from the catalytic center, and Gln188 and Glu190 are positioned toward the substrate. Therefore, although not having been identified with the afore-mentioned structural residues, Lys36 contributes to the proper function of the enzyme. While the lysine is structurally supportive in a local sense, it has a destabilizing effect on overall protein thermal stability. All Lys36 variants, except for LarvinA K36Q, resulted in higher $T_m$ values. The largest increase in thermal stability was seen in LarvinA K36E which showed a 1.5-fold increase in substrate affinity. The negative charge is, therefore, better tolerated at this position in terms of stability and substrate-binding, but it mildly impairs the catalytic function of the enzyme.

Variants with differing charges at position 36 were investigated to elucidate the role of Lys36 in membrane translocation. Regardless of the charge, all full-length proteins showed the same dispersed phenotype within the cytoplasm. In contrast, reversing the charge at the position of interest in the N-terminally truncated proteins resulted in a different phenotype— the toxins appeared as large puncta within the cell. From computational studies, removing residues from helix 1 caused a global shift in the calculated protein dipole and pH-dependency (41). The data outlined above supports the importance of the $\alpha$1-helix in cellular toxicity; however, the role of protein dipole will require further investigation.
Chapter 6: Isolation and Characterization of *Varroa* Toxic Protein
6.1 Abstract

Varroa Toxic Protein (VTP) was recently characterized as a novel virulence factor in Varroa destructor parasitism (33). Attempts to purify the protein using a pET-28a(+) plasmid were unsuccessful, despite changing the location and length of the poly-histidin-tag. Instead, the protein was cloned into a pGEX vector and purified using a combination of affinity and size-exclusion chromatography, which yielded 6 mg of protein/l of culture. MALDI-TOF mass spectrometry results showed a molecular weight 414 Da higher than the expected 40926.97 Da. This increase may be in part due to bound glutathione. The secondary structure of VTP was analyzed using CD spectrometry and the online server, JPred4. Both methods predicted that VTP consisted mainly of α-helices and loop regions. The melting temperature of VTP-GST was found to be identical to that of the GST-tag, suggesting that VTP is not contributing to the stability of the protein. Finally, the metal-coordinating ability of VTP was tested against Ni\(^{+2}\), Zn\(^{+2}\) and Ca\(^{+2}\) and compared to the GST-tag. There was no measurable difference between the two proteins, meaning that any metal-binding was due to the GST-tag and not VTP.

6.2 Purification and Identification of Varroa Toxic Protein

VTP was previously purified using a pET-32a(+) vector in E. coli Transetta cells (33). Following this protocol, an attempt to purify VTP involved the use of a pET-28a(+) plasmid. Similar to the pET-32a(+) plasmid, pET-28a(+) uses an N-terminal His\(_6\)-tag for IMAC purification. Initial attempts to purify VTP in the pET-28a(+) plasmid, however, showed that the protein was either not binding, or binding very weakly to the column (data not shown). Despite reconfiguring the poly-His-tag and linker region, there was no improvement in protein yield. Instead, VTP was cloned into the pGEX-2T(TEV) plasmid. The protein was purified using a
glutathione Sepharose column in combination with size-exclusion chromatography. Purity was assessed at each step using SDS-PAGE analysis (Fig. 6.1). The final protein yield was 6 mg/l of culture. MALDI-TOF mass spectrometry yielded a molecular weight of 41341.6 Da, larger than the predicted 40926.97 Da. The increase could be due to bound glutathione which has a molecular weight of 307 g/mol. However, this still does not account for the difference of 107 Da.

Figure 6.1: Purification and identification of VTP-GST. (A) SDS-PAGE analysis of VTP fractions after IMAC (Lane 2) and size-exclusion chromatography (Lane 3); BioRad Precision Plus Protein Standards are shown in Lane 1. (B) MALDI-TOF analysis of purified protein revealed a single peak at a higher molecular weight of VTP-GST.
6.3 Folded Integrity and Thermostability of *Varroa* Toxic Protein

The folded integrity of VTP-GST was assessed using circular dichroism (CD) spectroscopy (Fig. 6.2). The dataset was then analyzed using the online tool CAPITO (CD Analysis & PlottIng Tool), which makes robust secondary structure predictions based on comparisons against datasets from proteins of known structure (43). This algorithm predicted that the VTP-GST protein was 39% α-helical, 14% β-strands and the last 43% was irregular, or loop regions. By comparison, analysis of the GST tag predicted that the protein was 20% α-helical and 13% β-strands. These differences suggest that VTP is most likely a predominantly α-helical protein. To predict the secondary structure of VTP without the GST-tag, further analysis was carried out using the online tool JPred4. This prediction server uses the JNet algorithm which couples multiple-sequence-alignments with PSI-BLAST and probability models to assign secondary structure (44, 45). This tool predicted that VTP consists only of α-helices and loop regions, similar to the CAPITO analysis (Fig. 6.3). Finally, the melting temperature of VTP-GST was analyzed using a StepOnePlus Real-time PCR system which yielded a $T_m$ of 53.2°C (Table 6.1). This was identical to that of free GST, which had a $T_m = 53.1°C$, implying that this value is more representative of the thermal stability of GST rather than VTP. The fluorescent signal from the unfolding of GST may be masking the unfolding event of VTP, either due to the difference in size between the two proteins or because VTP may have a limited hydrophobic core region for the dye to bind. Alternatively, VTP may be unstable given that it failed to purify with a poly-His-tag, meaning it would not give a measurable signal in the SYPRO Orange thermal shift assay.
Figure 6.2: Folded integrity and thermal stability of VTP-GST compared to GST. (A) Circular dichroism spectra of VTP-GST and GST. Each curve represents an average of nine scans. (B) Derivative fluorescent curves for VTP-GST and GST, in which the curve minimum represents the respective T_m. Each curve represents an average of three thermograms using the SYPRO Orange™ thermal shift assay.

Figure 6.3: JPred4 analysis of the secondary structure of VTP. The VTP sequence is shown in bold and numbered. The JPred4 predicted secondary structure is depicted below, with helices depicted with an ‘H’ and loop regions depicted with ‘-‘.
### Table 6.1: $T_m$ values derived from the SYPRO Orange thermal shift assay.

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<th>Protein</th>
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<td>VTP-GST</td>
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<tr>
<td>GST</td>
<td>53.1 ± 0.6</td>
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</table>

### 6.4 *Varroa* Toxic Protein metal-coordination

VTP is homologous to a member of the neuroligin family, which have been previously characterized as calcium-binding proteins (33, 34). It also contains four cysteine residues and three histidine residues, which are commonly used to coordinate metal ions (46, 47). The metal binding properties of VTP were investigated using an IMAC column charged with either Zn$^{+2}$, Ni$^{+2}$ or Ca$^{+2}$. It has been previously reported that GST proteins can bind metal ions, so the GST tag was also tested as a control (48). There was no discernable difference between the metal-binding of VTP-GST and that of the GST tag against the three tested ions (Appendices, Fig. A.1). It is possible that the four cysteine residues perform another role within VTP, for example, forming disulfide bonds or behaving as catalytic residues. Disulfide bonds may be required to stabilize the tertiary structure of VTP or, if catalytic, this may indicate that VTP behaves as a cysteine protease or dehydrogenase (46).
Chapter 7: Conclusions and Future Directions
7.1 LarvinA

7.1.1 Conclusions

LarvinA was identified \textit{in silico} and characterized by genetic methods (22); work reported in this thesis has shown LarvinA to be a functional C3-toxin. It shares 99\% sequence identity with the truncated C3larvin, suggesting that LarvinA is the active form of the toxin. Kinetic comparison between these proteins revealed that LarvinA is 200-fold more active as an enzyme than C3larvin with respect to GH activity, and 12-fold higher with respect to the transferase reaction. While both proteins were equally cytotoxic when expressed in yeast cells, only LarvinA was able to successfully penetrate the membranes of macrophage cells. The catalytic activity and membrane translocation of LarvinA were superior to that of C3larvin, proving the importance of the N-terminus in proper toxin function.

The role of the N-terminus in enzymatic function and protein stability of LarvinA was investigated using a series of deletions. Removal of the first 21 residues did not impact the protein thermal stability ($T_M$); however, deletions from residues 21 to 34 resulted in lower thermal stability. Additionally, all variants had decreased $k_{cat}$ values ranging from a 3- to over a 37-fold reduction in catalytic activity. These experiments identified Ala31 as a critical residue in the correct orientation and packing of catalytic motifs within the active site. These findings were corroborated by the kinetic data from both F24A/A31L/W34A and A31L variants. Lastly, the removal of Asp23 significantly reduced the affinity of the protein for the $\beta$-NAD$^+$ substrate. Again, these findings were corroborated by the kinetic data of D23A/K25A/D27A/R28A and D23A variants. Analysis of the LarvinA homology model revealed that this residue was likely contributing to the proper orientation of the S-T-S motif. These findings elucidated novel
interactions within the α1-helix and ARTT-loop of LarvinA that contribute to NAD⁺ substrate-binding and catalysis.

Structural interactions between helix 1 and the active-site of CT-toxins had been previously investigated (41). A computational study identified Ile153 and Tyr178 as key structural residues responsible for the proper interaction and orientation of the α1-helix and catalytic motifs (41). Experimental data confirmed this structural classification, since both Ile153A and Tyr178A variants had reduced thermal stability and catalytic activity. Interestingly, close in space to the conserved tyrosine was Lys36– the only residue difference between the shared sequence of C3larvin and LarvinA. A 3-fold reduction in enzymatic efficiency was reported when Lys36 was substituted with an alanine residue. Lys36A yielded an increased $T_m$ value, suggesting that while the lysine is structurally important for enzymatic function, it is destabilizing to the protein structure. This lysine residue is conserved amongst C2-toxins but not in C3-toxins. Consequently, this novel interaction will require further investigation in both toxin subgroups.

The importance of the N-terminus to cell entry has been previously described (16); however, its specific contribution is unclear. Through fluorescence microscopy experiments, it was shown that the removal of the first 34 residues of the N-terminus had no effect on cell entry and localization. All deletions were phenotypically similar to WT and appeared dispersed within the cytoplasm. Investigation into the only residue difference between C3larvin and LarvinA ΔY2-W34 found that the charge reversal at this position (position 36 in the non-truncated protein and position 2 in the truncated protein) from positive to negative resulted in a differing phenotype where the toxin appeared as large puncta. The reversal and removal of charge in the non-truncated toxins, however, had no effect on cellular phenotype. Therefore, it was the combination of the
truncated N-terminus and the substitution of Lys36 (position 2 in the truncated protein) that caused the altered phenotype. This suggests that the difference in cell entry is not based on a single residue but may instead be based on a global conformational change within the protein. Computational studies have revealed a significant difference in protein dipole and pH-dependency upon the removal of the N-terminus. The importance of protein dipoles in cell entry will require further investigation. Finally, co-localization studies found that C3larvin failed to target cellular RhoA, suggesting that it is retained within the vesicle system upon cell entry. This could explain how cells treated with C3larvin remain unaffected, despite C3larvin targeting RhoA in vitro, and being lethal to yeast cells when expressed in vivo (16). When considered with earlier findings, these results suggest an importance of protein charge in the cellular pathway taken by C3-toxins when intoxicating a target cell. This will require further studies.

7.1.2 Future directions

7.1.2.1 Crystallography

The work described in this paper has focused on the kinetic characterization of LarvinA; however, many future avenues of research remain. Firstly, the structure of LarvinA has yet to be elucidated. Crystallography experiments thus far have not generated any reproducible protein crystals and, therefore, should continue. These experiments, while useful to confirm overall protein topology, would be specifically important for determining the structure of the N-terminus. It may also give insight into the orientation of the N-terminus; specifically, whether it is extended away from the protein or held against it. Moreover, co-crystallizing LarvinA with an NAD$^+$ analog could show specific differences between the active site of LarvinA to that of other C3 toxins. Based on the catalytic investigation of LarvinA, it appeared that the catalytic arginine behaved differently
to that of other C3 toxins. It would be beneficial to examine the orientation of the NAD$^+$ substrate within the active site to describe novel interactions.

**7.1.2.2 Investigation into the dual C2-/C3-character of LarvinA**

LarvinA is functionally related to C3-toxins; however, it also shares C2-like toxin characteristics. For example, the LarvinA gene, c3larvinA, was found alongside a gene encoding a translocating B-domain within the *P. larvae* genome, and LarvinA also contains residues that are conserved within the C2-subgroup (22). One of these shared residues, Lys36, is functionally important to the catalytic ability of LarvinA. These instances of dual-character should be further explored as possible sites of interest. Investigation into these residues, both computationally and experimentally, may reveal significant details about the evolution of both toxin classes or may result in the re-classification of the current CT-family subgroups. Alanine-variants of LarvinA and C3bot1 should be characterized in terms of protein stability and catalytic efficiency and should be compared to draw parallels. This can also be extended to other C3-toxins and the C2-subgroup.

**7.1.2.3 Cell localization studies**

This work has elucidated a possible role of charge or protein-dipole in cell intoxication. Future work should examine the cellular localization of LarvinA ΔY2-W34 variants, with alanine, glutamine and arginine substitutions at position 2. This will help determine whether a specific charge or residue is needed at this location. Additionally, it is unclear why C3larvin fails to localize to RhoA in the target host cell. Endosomal markers could be used in fluorescence microscopy experiments to determine if the protein is being retained within the endosomal pathway. This would also help determine whether C3larvin is trapped in early or late endosomes and would facilitate tracking the movement of the toxin within the cell. Finally, live-cell imaging
using fluorescently-labelled toxins and organelles can be used to better characterize the cellular pathway taken by C3-toxins.

7.2 Varroa Toxic Protein

7.2.1 Conclusions

VTP is a newly discovered virulence factor from the ectoparasite, *Varroa destructor* (33). Various attempts to purify the protein using a poly-histidine-tag were unsuccessful, regardless of tag length or placement. VTP was instead cloned into a pGEX-2T-TEV vector, and purified using a combination of affinity and size-exclusion chromatography. This purification scheme yielded 6 mg of protein/l of culture. The CD spectrum of VTP-GST showed similar secondary structure patterns to that of the GST-tag, but contained more α-helical content. These results combined with the JPred4 analysis indicate that VTP is a largely α-helical protein. There was no difference in T\textsubscript{m} values between VTP-GST and GST, suggesting that VTP is not contributing to the thermal stability of the protein. Since VTP was unable to purify without a large, solubilizing tag, it stands to reason that VTP is likely unstable and not folded properly. Finally, the metal-binding abilities of VTP-GST were investigated to explore the role of the four cysteine residues. Preliminary results showed no difference between the metal-coordination of VTP-GST and the GST-tag; however, this will require further investigation.

7.2.2 Future directions

VTP represents a novel virulence factor in *Varroa destructor* and could be a viable target for therapeutics against *Varroa destructor*. Currently, there is little known about the structure and function of this protein, how it causes larval death in *Apis cerana* or how it increases the frequency
of deformed wing virus in *Apis mellifera*. Therefore, there are various avenues of research for future characterization.

### 7.2.2.1 Crystallography

VTP has a single homolog with which it shares only 29% homology. There are no structures that it can be compared to, and therefore there is little one can say about its overall folding. Elucidating the structure of this protein would be a useful step towards identifying possible sites for interactions with other macromolecules, or possibly beginning to identify catalytic mechanisms through previously described active-site topologies. Moreover, using crystallography, one could also determine whether the protein requires bound divalent cations. Finally, describing the structure of this protein would be beneficial to begin identifying possible inhibitors. By identifying regions associated with function, small molecules can be chosen or designed to target these areas and prevent VTP from interacting with any binding partners. Any inhibitors found could be used as a treatment to protect against the effects of the *Varroa* mite.

### 7.2.2.2 *In vivo* studies

It is critical to understand the biological function of VTP to fully understand *Varroa* parasitism. Results from Zhang and Han show a dramatic relationship between VTP treatment and mortality rates in *A. cerana* and DWV rates in *A. mellifera* (33). Based on the relationship between VTP and DWV titers, it seems likely that the protein has immune-modulating capabilities. Previous work has shown that mite parasitism and injection of crude mite lysate causes a significant decrease in haemocyte concentration and prophenol oxidase gene expression (49). It has also been documented that treatment with mite saliva causes damage to haemocytes, often leading to cell lysis (27). To date, the underlying cause for these effects have not been described.
Future studies should examine the possible role of VTP in haemocyte damage and gene expression, with specific interest in genes associated with immunity.
REFERENCES


A.1: Methods

Table A.0.1: Primer sequences for each catalytic variant.

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<tr>
<th>Primer sequence</th>
<th>Type</th>
<th>Catalytic Variant</th>
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<td>5’ CCAGTATAAGGGTGCGTATGCGCTGCTGCTGCCG</td>
<td>Forward</td>
<td>QxE</td>
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<td>5’ GCAGCAGGCCATACGCACCCTTATACTGGCTAATTTTATC</td>
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<td></td>
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<tr>
<td>5’ GGTTATCTGGCCGCCGCCATTGTGAGTAATCAGCAGTTTG</td>
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<td>STS</td>
</tr>
<tr>
<td>5’ CCATATTTCGTTACGAATTTCG</td>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>5’ CGTGCAAGGTGTATGCGGGCGATGATACCAGTATTTTTG</td>
<td>Forward</td>
<td>R120</td>
</tr>
<tr>
<td>5’ GGTATCATCGCCCCGCATACACCTGCACGTTTCGCTCA</td>
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Table A.0.2: Primer sequences for each N-terminal variant.

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<tr>
<th>Primer sequence</th>
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<tr>
<td>5'- GCAAGGCGCCCTGGAAAGGGGTTAAAAAG</td>
<td>Forward</td>
<td>F42A/A49L/W52A</td>
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<tr>
<td>5' - CGATCTTCTTTGACATCCTTTGTC</td>
<td>Reverse</td>
<td></td>
</tr>
<tr>
<td>5' - CAGCGCAGTTTTAAAGAGGATCCTGCAAGGCGCGA</td>
<td>Forward#1</td>
<td>D41A/K43A/D45A/R46A</td>
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<td></td>
</tr>
<tr>
<td>5' - CCGTTATCTTTCACTTCAAAACGATTATAGC</td>
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<tr>
<td>CCGCAGCGCCAAAGGTTTTAAAGAGGATCCTGCAAGGCGCG</td>
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<td>D41A</td>
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<tr>
<td>5' CGGTATCTTTCTACCTTCAAAACGATTATAGC</td>
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<td></td>
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<tr>
<td>CGCAAAGCCCTTGGAATGGGTAAAAAGGATAATAAAGA</td>
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<tr>
<td>GGTATCATGCAGCGAGTACTACCTGAGCTGTCGCTTCGCTC</td>
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<tr>
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### Table A.3: Primers designed for LarvinA N-terminal deletions.

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</thead>
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<td>Reverse</td>
<td>-</td>
</tr>
<tr>
<td>5’ GATCCATATGAAAGATTGTAAAGAAGATCGCAAAGCCGCC</td>
<td>Forward</td>
<td>Y20 – A39</td>
</tr>
<tr>
<td>5’ GATCCATATGTTAAAAGAAGATCGCAAAGCCGCCGAAAAA</td>
<td>Forward</td>
<td>Y20 – D41</td>
</tr>
<tr>
<td>5’ GATCCATATGGAAGATCGCAAAGCCGCCGAAAAATGGGG</td>
<td>Forward</td>
<td>Y20 – K43</td>
</tr>
<tr>
<td>5’ GATCCATATGGCGAAAAATGGGGTAAAAAAGAATATAAA</td>
<td>Forward</td>
<td>Y20 – A48</td>
</tr>
<tr>
<td>5’ GATCCATATGCGAAAAGCCGCCGAAAAATGGGGTAAAAA</td>
<td>Forward</td>
<td>Y20 – D45</td>
</tr>
<tr>
<td>5’ GATCCATATGTTGGGTTAAAAAGAATATAAAGCCTGGA</td>
<td>Forward</td>
<td>Y20 – K51</td>
</tr>
<tr>
<td>5’ GATCCATATGTTGGGTTAAAAAGAATATAAAGCCTGGA</td>
<td>Forward</td>
<td>Y20 – W52</td>
</tr>
</tbody>
</table>
A.2: Varroa Toxic Protein

**Figure A.1: SDS-PAGE analysis of the metal-binding abilities of VTP-GST.** An IMAC column was charged with 0.1 M of Ni$^{2+}$, Zn$^{2+}$ or Ca$^{2+}$. VTP-GST or GST was loaded onto the column before being washed with equilibration buffer (50 mM Tris-HCl, 1 M NaCl, pH 7.4) and then elution buffer (50 mM Tris-HCl, 1 M NaCl, 50 mM EDTA, pH 7.4). Fractions were analyzed with SDS-PAGE. (A) Nickel-binding assay showing the elution fractions of VTP-GST (Lane 2 – 5) against the elution fractions of GST (Lane 6 – 9) compared against BioRad Precision Plus Protein Standards (Lane 1). (B) Zinc-binding assay showing the elution fractions of VTP-GST (Lane 2 – 5) against the elution fractions of GST (Lane 6 – 9) compared against BioRad Precision Plus Protein Standards (Lane 1). (C) Calcium-binding assay showing the wash fractions of VTP-GST (Lane 2 – 5) against the wash fractions of GST (Lane 6 – 9) compared against BioRad Precision Plus Protein Standards (Lane 1).