The characterisation of GNA based fusion proteins and their potential as a novel class of bio-insecticides.

PINCHES, EDWARD,DAVID

The characterisation of GNA based fusion proteins and their potential as a novel class of bio-insecticides.

Name: Edward Pinches

College: St Mary's College

Degree: MRes

Department of Biosciences

Year of Submission: 2018

Supervisor: Dr E Fitches

Abstract

The human population is predicted to surpass 9 billion by 2050, and producing enough food remains a global challenge. Food security is paramount; with 16% of all crop losses due to insect pests and current chemical control agents for these species largely non-specific, new classes of novel insecticides are needed.

ω-Hv1a-ACTX (Hv1a), a peptide from the Blue Mountain funnel web spider (*Hadronyche versuta*) has been fused to the plant lectin *Galanthus nivalis* agglutinin (GNA). GNA acts as a 'carrier' molecule allowing venom derived peptides to be delivered to the insect haemolymph and reach their site of action. The resulting fusion protein Hv1a-GNA is toxic orally towards Lepidopteran, Coleopteran and Hemipteran pests but displays no toxicity towards honeybees (*Apis mellifera),* a beneficial pollinator species.

In this study the expression profiles of four Hv1a/GNA based fusion proteins produced in the yeast *Pichia pastoris* were assessed. The oral toxicity of the Hv1a/GNA fusion proteins to *Acyrthosiphon pisum* (pea aphids) was established and LC50 values calculated. Proof of concept was provided for transiently expressing a GNA/Hv1a fusion protein in tobacco (*Nicotina benthamiana*) and future work to produce of stable expressing *Arabidopsis thaliana* lines is recommended.

The first ever double toxin-GNA fusion protein (DT1) containing the spider toxins Hv1a and ω-Hv1a-Hv1g (Hv1g) was purified from fermented culture supernatant. It was shown that DT1 exhibits toxicity by injection to *Mamestra brassicae* and LD50 values were calculated. This work proved that both toxins within the fusion protein are active.

Results in this thesis demonstrate the importance of developing fusion protein based biopesticides, which will be essential for protecting crops future.

Table of contents **Page**

Acknowledgments

I would like to thank Dr E Fitches (Department of Biosciences, Durham University) for providing not only an interesting project but also for her support and guidance throughout my time at Durham particularly during this Masters project.

Thanks to all the members of Lab 1, for providing such a good working, supportive and friendly environment. Particular thanks must be given Dr Prashant Pyati, Dr Min Cao, Mr Jake de Thier, and Miss Caitlin Willis for their invaluable help, kindness and support. Further thanks go to Ms Michelle Powell, Ms Dania Martinez and Dr Zhaou. You have made my Masters truly an experience I won't forget.

Thanks also to Dr Prashant Pyati and Miss Becky Morrell who produced the expression constructs or produced much of the fusion protein powder to be subsequently used. Thanks to all members of Lab 6/7 whose expertise proved invaluable.

I am very grateful for the funding provided by the N8 research council that enabled me to carry out this work. I would like to extend this thanks to St Mary's College, Durham that offered partial financial assistance.

Finally, I would like to thank those closest to me, my family and friends especially for their support throughout this project

Edward Pinches

List of abbreviations.

Amino acid Code

Full Name	Abbreviation (3 Letter)	Abbreviation (1 Letter)
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Nucleic acid abbreviations
A - Adenine
C - Cysteine
G - Guanine
T - Thymine

Additional Abbreviations
aa- amino acid
AOX1- Alcohol oxidase 1
bp - base pairs
°C - degrees Celsius
CBB - Coomasie brilliant blue
CNS - Central nervous system

dATP: deoxyadenosine triphosphate
dCTP - deoxycytosine triphosphate
DDT - Dichloro-diphenyl-trichloroethane
dGTP - deoxyguanine triphosphate
DNA - deoxyribonucleic acid
dNTPs - deoxyribonucleotide triphosphate
DT1 – Double Toxin 1
dTTP - deoxythymine triphosphate
EU - European Union
FAO - Food and Ariculture Organisation
FERA - Food and environment Research agency
g – gram
GM - Genetically modified
GNA - *Galanthus nivalis* agglutinin
GSP - Gene specific primer
Hv1a - ω-Hv1a-ACTX
Hv1g- ω-Hv1g-ACTX
kDa - KiloDalton
LSLB - low salt Luria- Bertani broth
M – Marker (SDS7)
nm- Nano metre
OD - Optical density
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PIs - Protease inhibitors
RT- run through
Rpm- Revolutions per minute
S- seconds
SDS7 - Sodium dodecyl sulphate 7 Marker
SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPB - sodium phosphate buffer
TAE - Tris acetate EDTA buffer
TEMED - Tetramethylethylenediamine
UN - United Nations
W- Binding buffer wash
YPG - Yeast Extract Peptone-Glycerol

List of figures.

Chapter 5

Chapter 6

Chapter 7

List of tables

Chapter 1

Chapter 3

Chapter 5

Chapter 7

Chapter 1

1. Introduction

1.1 Is there a global food security crisis?

The human population is growing exponentially; it is projected to reach 9.7 billion by 2050 and will result in a substantial increase in demand for food (UNDESA, 2015). Coupled with other threats posed by climate change, resource scarcity and crop losses to name but a few, there is severe pressure on the international community to increase global crop production (Calzadilla et al., 2013; FAO, 2011).

In 2001, the State of Food Insecurity labelled "Food security [as] a situation that exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life." (FAO, UN 2001). In order to ensure food security for the additional 2.3 billion people the FAO stated that by 2050 food productivity must increase by 70 per cent (FAO, 2009).

Basic food prices are on the rise and are set to double by the year 2050, prices spiked in 2007-2008 and this brought a huge international focus on the issue of food security (IPCC, 2014). In 2014-2016, there were 795 million undernourished people in the world, with an additional 40 million people pushed into poverty by the rise in cereal prices (FAO: 2012, 2014). Furthermore, high food prices have had a large impact on the types of food consumed resulting in malnourishment and other underlying health problems in some areas (Green et al., 2013). It is therefore of paramount importance that the maximum yield of quality product is obtained from the crops grown around the world.

1.1.1 The effects of climate change on food security.

Current global changes are further stretching the demand for increased food production. Climate change will have complex effects on crop growth, yield and distribution (Schmidhuber and Tubiello, 2007). Researchers have concluded that the effects of climate change are 'very likely to increase the risk of hunger' and increase food market volatility (Schmidhuber and Tubiello, 2007).

Average atmospheric temperature is predicted to rise by two degrees by the end of the century (Stocker et al., 2013). Most models also predict an average increase of atmospheric carbon dioxide (CO_2) concentrations, from 391ppm in 2011 to in excess of 500ppm by 2100 (Solomon et al., 2007). Changes to precipitation patterns, will increase the regularity of extremes in weather, and increase the likelihood of droughts and flooding which will have an overall negative effect on crop yields (Chakraborty and Newton, 2011). The changing climate will alter

the natural constraints on food production, providing an increased area for crop production in northern latitudes, but reducing the area available in the tropics (Zabel et al., 2014). Ultimately these changes will likely mean that an increased yield from current producers is viable, but at the cost of losing suitable land for new crops to be planted (Zabel et al., 2014).

Insect pests and other pathogens will also be greatly affected by climate change (Hullé et al., 2010, Bale et al., 2002, Mcvean, and Dixon, 2001, Woodman, 2015). Although it is not yet possible to model accurate population predictions for all insect species, a detrimental impact on food yields is likely (Hullé et al., 2010). Increasing temperatures are widely accepted to be the most crucial factor affecting insect pests with many believing it to be far more important than any other environmental factor. Effects are thought to include the expansion of insect ranges and shorter generation times (Bale et al., 2002). Shorter generation times and high reproductive rates, mean that insects can respond more quickly to changes in climate, than longer living organisms such as the plants on which they feed (Bale et al., 2002).

Whilst there are fewer studies assessing the effects of increased precipitation rates on insects and their impact as pests, increased incidents of extreme conditions remains important. Pea aphids for example, are not suited to long periods of drought, whilst flooding will have a detrimental impact on insects whose overwintering eggs and/or pupae are left in the soil (Mcvean, and Dixon, 2001; Woodman, 2015). Meta-analysis also shows that elevated CO_2 levels cause an elongated developmental time and a decrease in abundancy in the range of insect species (Stiling and Cornelissen, 2007).

The changing climate has and will continue to affect the amount and regions of land that are suitable for crop production. Not only will global warming have an effect on the world's climate and weather patterns, but also on other natural resources including the worlds fresh water supply.

1.1.2 Changing land-use and the effect on crop production

1.1.2.1 Urbanization

Climate change is not the sole contributor to changes in the amount of land suitable for good quality crop production. Increased industrialisation coupled with the expanding urban population, which is projected to grow by more than a billion people between the years of 2010 and 2025, will put more pressure on crop producers (United Nations, 2008). This pressure is intensified by a sharp reduction in the rural population that is predicted to fall by nearly 200 million by 2050 (FAO, 2017).

Currently only 25% of the earth's terrestrial surface is cultivated, and urbanization continues to limit the areas available to produce food (Hassan et al., 2009). This loss of land is sometimes exaggerated with a study finding only 1% of the western world is classed as urban (Schneider,

Friedl and Potere, 2009). Yet conflicting studies find that in certain industrialised countries, including the US and countries within the EU, urbanized land equates to almost 31 million hectares, an area in excess of two times greater than the amount of land lost to biofuel production (14 million hectares) (Langeveld et al., 2014). However, it is clear that continued urbanization will greatly affect the agricultural industry in the years to come.

1.1.2.2 Crops for biofuel or for food?

Whilst the demand for global cereal production is set to rise from 2.1 billion to 3 billion tonnes by 2050, globally the demand for transport fuel is set to surpass this (FAO, 2009). Growing crops for biofuels is already a major industry in Brazil, United States and Europe, with Malaysia and Indonesia beginning to take advantage of new markets (Tenenbaum, 2008).

In America the production of biofuel accounted for a 20–25% increase in maize prices between 2001 and 2007 (Zilberman et al., 2012). However, the direct association between the amount of land lost to biofuel production and the increase in food prices is still contested, with only a weak correlation found in a large New Zealand study ((Mohr and Raman, 2013; Araujo et al., 2017; Zhang et al., 2010).

1.1.2.3 The trade-off between crop production for animal feed and humans

Animals are an important food source and contain micronutrients that are difficult to obtain from plant based foods alone (Murphy and Allen, 2003). Currently there are around 1.5 billion cattle worldwide and this figure is expected to increase to 2.6 billion by the year 2050 (FAO, 2009). There is an increasing amount of land dedicated to livestock and animal feed. Within developing countries, the share of the use of cereals for animal feed doubled from 1980s to 1990s (Delgado, 2003). Increasing land-use for animal feeds has been criticised as it directly competes against crops grown for human consumption.

The trade-offs between land used for food, feed and fuel require further research as substantial evidence gaps remain (Manceron et al., 2014). Although whilst it is clear that there is increasing competition from all three facets, there is uncertainty over many of the estimates made and a refinement of models is needed (Manceron et al., 2014).

1.2 Agricultural losses due to insect pests

Crop destruction by insect pests is a formidable problem and accounts for up to 16-18% of total crop loses worldwide (Oerke, 2006). Even with effective control measures in place, insect pests still contribute to between 7.9% and 15% of losses to the major crops wheat, rice, and cotton (Oerke, 2006). In the United States, the major destruction of crops by insects, weeds and disease equates to approximately 244 billion US dollars ($) in losses per annum (Gatehouse et al., 2011). This is despite over $10 billion being spent on chemical insecticides annually (Grube et

al., 2011). Without the use of insecticides, it is estimated that 78% of all fruits, 54% of vegetables, and 32% of cereal crops would be lost to insect pests (Cai et al., 2009).

Crop losses due to insect pests is a complex phenomenon; plants and insects have co-evolved over 400 million years. An extraordinarily diverse evolutionary relationship between both plants and insects exists, owing to a continued 'arms-race' (Labandeira, 2013). This diversity allows insects to damage and destroy crops, not only by manual destruction of leaf tissue through feeding but also by consuming fruiting bodies, roots or even directly removing nutrients through the phloem. Not only can insects cause major physical damage, they are also major vectors for plant disease (Bar-Joseph et al., 1979). These viral diseases are significant and account for up to 47% of the new diseases effecting plants (Anderson et al., 2004).

Insects are not only major vectors for plant diseases but they are also major 'carriers' for human diseases; Malaria, Yellow Fever and Zika included (Vaughan et al., 1999, Tolle, 2009). Livestock populations are also significantly affected by vector borne viruses carried by insects which account for 29% of the known mammalian viruses (Olival et al., 2017). Therefore control of insect populations would be beneficial, not just for crop protection but also for the agricultural industry and the human population as a whole.

1.2.1 Hemipteran pests

Hemipteran pests, are commonly known as the sap-sucking insects and include aphids and whiteflies. There are estimated to be 4,400 aphid species, many of which are agricultural and ornamental pests (Blackman and Eastman, 2000). Aphids affect plant growth both directly, through feeding on phloem sap, but also indirectly, by vectoring of plant viruses which account for a substantial proportion of crop losses (van Emden and Harrington, 2007).

1.2.1.1 *Acyrthosiphon pisum*, as a Hemipteran pest and their use in research

Acyrthosiphon pisum, commonly referred to as pea aphids feed specifically on legume plants and are a species genetically very closely related to another important crop pest, the peach potato aphid, *Myzus persicae* (von Dohlen et al., 2006). *Acyrthosiphon pisum* are found worldwide in temperate climates, not only are they of major agricultural importance but they are also widely used as the model system for aphid species in laboratory experiments (van Emden and Harrington, 2007; Yang et al., 2014). *Acyrthosiphon pisum* have been used in this thesis to investigate the oral toxicity of fusion proteins.

Considered large amongst aphid species, pea aphid adults can reach up to 4mm in length, and are stylet feeders (Adams et al., 1996). Generally, they are found on the underside of plant leaves, using their stylets to feed on the phloem sap which is rich in sugars but not in all essential amino acids (figure 1.1). However, an intracellular mutualistic symbiotic relationship

with *Buchnera aphidicola,* provides essential amino acids allowing *A.pisum* to overcome the nutritional deficiencies in phloem sap (Moren et al., 1993).

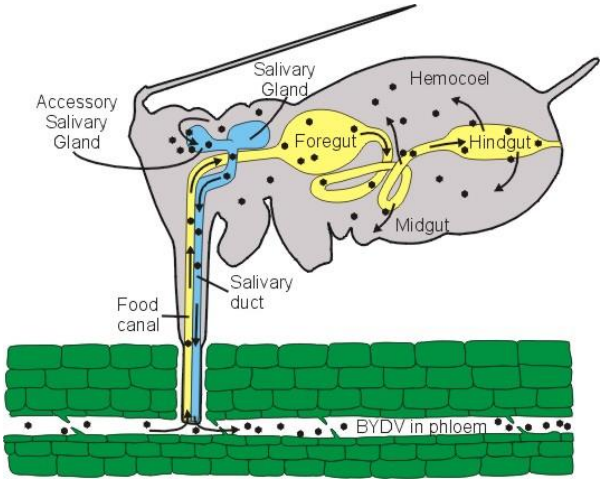

Figure 1.1 Diagram of an aphid feeding on phloem sap containing free amino acids using the stylet apparatus. Figure taken from D'Arcy, C.J. and L.L. Domier, 2000.

1.2.1.2 *Acyrthosiphon pisum*, life cycle

Acyrthosiphon pisum have high reproduction rates and have evolved a complex life cycle displaying much phenotypic plasticity (Blackman and Eastop, 2000). *Acyrthosiphon pisum* are viviparous and undergo parthenogenesis during the warmer spring and summer months. During the colder winter months egg laying females are produced and males inseminate them; eggs are subsequently laid and take 100 days to fully develop (Blackman and Eastop, 2000). In contrast, parthenogenetic embryogenesis is rapid and completed in around 10-12 days. Many laboratory strains are situated where there is no prolonged period of cold and undergo continuous cycles of asexual reproduction. Offspring undergo four moults during larval development to become either winged or non-winged adults, the latter are able to move and infect new plants. Winged individuals are produced when crowding becomes a problem or when there is stress during prenatal stages (Powell et al., 2006).

1.2.2 Lepidopteran Pests

The lepidopteran order is the second largest order of insects, and are found on every continent bar Antarctica (Scoble, 1992). The larvae of Lepidopteran insects, many of which are polyphagous, are major agricultural pests and exert damage mainly through feeding on leaf tissue (Cates, 1980).

1.2.2.1 *Mamestra brassicae* - a major Lepidopteran pest.

Mamestra brassicae have a wide geographic range and are found in Europe and in the greater part of Asia, although it has been noted that they are becoming less common in the north of Britain (Pollini, 2006; Goulson, 1994). *Mamestra brassicae* are highly polyphagous with larvae

feeding both day and night on over 70 host species (Rojas et al., 2000). Primarily feeding on *Brassica* species, *M. brassicae* also feed on sugar cane, lettuce and potatoes (Rojas et al., 2000). Not only do they exert damage by feeding on the leaf tissue but their excrement on both flowers and leaves reduce the quality of the crop (Pelosini, 1998). Furthermore *M. brassicae* larval feeding indirectly leads to an increase in pathogenic fungi and bacteria that infect the plant on which they feed (Corvi and Nardi, 1998). Although *M. brassicae* can successfully be treated with insecticides they are still major crops pests, with insecticides only effective against smaller larvae (Johansen, 1997a).

1.2.2.2 Life cycle of *Mamestra brassicae* and use in research.

Mamestra brassicae have previously been used in the laboratory to assess the biological activity of recombinantly produced toxins and fusion proteins (Fitches et al., 2012). In this thesis they are used to assess the toxicity of fusion proteins. It is particularly important to understand their life cycle as conventional insecticides are found only to be active against young and small larvae; with the literature suggesting that optimal efficiency of insecticides is when larvae are up to 12mm in size (Johansen, 1996, 1997a).

Their pale green eggs, which darken as the *M. brassicae* larvae develop, are laid in early May or June by female adult moths in batches of 60 or more, generally on the underside of leaves. Larvae develop through a series of molts known as instars, of which there are 5, I-V, with fifth instar larvae displaying negative phototaxis whereby they move towards the centre of the plant (Omino et al., 1973). Fifth instar stage can be visualised easily as *M. brassicae* larvae show a wide difference in the amount of melanisation, particularly during the this instar stage (Burov and Mokrousova, 1970). Mature larvae then pupate in the ground at a depth around to 2-4 cm in August and September. Diapausing pupae hibernate with adult moth emergence in June when mating occurs eggs are laid and the cycle repeats (Devetak et al., 2010).

1.3 Combating crop losses due to insect pests

Although insects control is deemed vital, insects make up a critical part of the ecosystem. Aphids for example appear in many food chains, and regulation of aphids often ecologically kept in check by other insects. Insects can also play a key role in plant defence against other pests and diseases and broad bean plants with *A. pisum* feeding on are six times more attractive than uninfested plants towards the parasitioid *Aphidius ervi* (Guerrieri et al., 1993*)*. Despite this insects can be serious pests, and treatments are required to save crop yeidl.

Currently combating crop losses by insect pests has primarily relied on chemically synthesized insecticides, with mixed success. The crop protection market as a whole, is predicted to reach 71.3 billion US dollars by 2018, with over $6 billion being invested annually into research and development of pesticides as of 2012 (TransparancyMarketResearch, 2012).

1.3.1 Chemical Control Measures

A large proportion of current chemically synthesized insecticides, mimic the action of insect neurotransmitters and act primarily on four nerve targets; acetylcholinesterase, voltage-gated ion channels, the nicotinic acetylcholine receptor (nAChRs), and the gamma-aminobutyric (GABA) acid receptor.

1.3.1.1 Neonicotinoids

Neonicotinoids are a class of chemically synthesized chemicals similar in structure to nicotine and act as neurotoxins, interacting with nAChRs in the insect with lethal and sub-lethal effects on honeybees (*Apis mellifera*) (Bryden et al., 2013, Cresswell, 2010)

Electrophysiological studies show that neonicotinoids act as agonists on nAChRs, opening cation channels, with disruption to voltage-gated calcium channels also contributing to their insecticidal activity (Jepson et al., 2006). As a result, their agonistic action induces continuous excitation of the neuronal membranes, producing discharges leading to paralyses and cell energy exhaustion. However, the development of insecticide resistance against neonicotinoids has been observed in the brown planthopper (*Nilaparvata lugens*) and has since been found in other Asian countries (Harris, 2006).

The differences in structure of between insect and mammalian nACHR subunits can partially explain the selectivity of neonicotinoids to arthropods and the supposed relatively low toxicity to vertebrates (Tomizawa and Casida, 2003). However, neonicotinoids have both lethal and sub-lethal effects on *Apis mellifera*. *Apis mellifera*, the honeybee, is a useful plant pollinator, and with 35% of the world's crop production relying on insect pollinators, the specificity of insecticides is vital. (Klein, et al., 2007). This non-specificity is a major drawback to neonicotinoids particularly because heavy use of the insecticides coincides with flowering and a marked reduction in pollinator populations is seen (Nicholls and Altieri, 2013). Neonicotinoids are in the process of being banned within the European Union, however the cost to companies for developing novel insecticides is high and the rate of development is slow.

1.3.1.2 Pyrethroids

Pyrethroids are synthetic organic compounds synthesized from chrysanthemum. The insecticidal activity of pyrethroids depends on their ability to bind to and disrupt voltage-gated sodium channels of insect nerves (Soderlund, 2005). In general, pyrethroids have extremely high insecticidal activity at extremely low doses, maintaining biological activity over a long period of time. However, there is widespread agreement that voltage-gated sodium channels are also important targets for the neurotoxic effects of pyrethroids in mammals (Soderlund 2010b).

1.3.1.3. The limitations of chemical control measures

Despite their widespread application, chemical control measures have many limitations and their continued use remains controversial.

One of the most widely known organochlorine pesticides, Dichloro-diphenyl-trichloroethane (DDT), directly led to a 15-20 fold increase in the amount of pesticide used (Oerke and Dehne 2004). Despite the extensive use of DDT, there is evidence that DDT and its metabolite p,p-dichlorodiphenyldichloroethylene (DDE), may have carcinogenic effects, with both DDT and DDE exposure in-utero associated with neurodevelopmental defects in children (Eskenazi et al., 2006). Furthermore, it has also been shown that there is an ecological consequence to frequent use of DDT. However, it is no longer in use across the western world (Delaplane, 2013).

Resistance to insecticides is also becoming a major problem hampering global food security efforts. Since the first reported incidences of resistance to DDT in the 1940s and 50s (Livadas et al., 1953), the number of species resistance to one or more chemical pesticides has risen dramatically and now stands in excess of 1000 (Miller, 2004). Furthermore, resistance has now been recorded for all major classes of chemical compounds (Bass et al.,2015). Physiological resistance is thought to be due to the limited number of molecular targets for insecticides to act upon, with insecticidal resistance arising in various ways including enhanced metabolic detoxification in the insect, decreased target sensitivity and increased sequestration (Liu et al., 2006; Karunaratne 1993). Therefore, new classes of insecticides are needed to overcome these short falls.

Most chemical insecticides are water soluble, which helps aid application. Yet this increases the chance of insecticides leaching into water run offs, with concentrations in fish measured to be far greater than enforced limits (Liong et al., 1988). Indeed, metaldehyde, a widely used molluscicide has been detected in surface and in drinking waters in the UK above levels set for European Union statutory drinking water limit (Kay and Grayson, 2013). Many current water treatment technologies are inefficient at treating water contaminated with metaldehyde and the scientific community continue to study methods of metaldehyde removal (Busquests et al., 2014). Many difficulties in assessing the toxicity of insecticides to non-target organisms still remain (Atkar, 2009). Furthermore, although pesticides have, in the main, been designed not to harm human health, studies have revoked this (Forget, 1993; Nigam et al, 1993). Numerous scientific papers have suggested pesticides have negative health effects including dermatological, neurological and carcinogenic effects (Speiwak, 2001; Keifer and Firestone, 2007; Basil et al., 2007). In some studies it has even been suggested that extremely high doses can result in human death (Gunnel et al., 2007).

The issues outlined, coupled with public concern and pressure groups, have led to many chemical insecticides being removed from the market. Restrictions have been heavily imposed particularly within the European Union, which has one of the strictest regulations to chemical insecticides in the world, including directives 2009/128/EC and 91/414/EEC. Although the

benefit chemical pesticides have had since their introduction should not be overlooked; there is a clear need to develop non-chemical and more specific insecticides to tackle insect pests, and ensure future food security.

1.3.2 Non-chemical methods of insecticides

Multiple concerns with chemically synthesized insecticides, including enhanced insect resistance and problems with specificity have largely driven the development of non-chemical methods of insecticides (Chandler et al., 2012).

1.3.2.1 Protease inhibitors (PIs)

Protease inhibitors (PIs) are low molecular weight proteins which function by forming specific binding complexes with digestive proteinases blocking the active site of the target enzyme (Azzouz et al., 2005). Protease inhibitors are a well-established plant defence mechanism against predators and are one of the prime candidates to become the major class of bioinsecticides (Gatehouse, 2011). Protease inhibitors were first used in 1947, when a study found that the larvae of certain insects were unable to develop normally on soybean (Mickel and Standish, 1947). They act by blocking insect midgut proteinases, inhibiting protein digestion and this reduces the uptake of amino acids. This creates a nitrogen deficiency which means insects are unable to grow and ultimately die by starvation (Lawrence and Koundal 2002, Azzouz et al. 2005).

Protease inhibitors are made up by four basic families, based on amino acids in their reaction centre, and are classified as either serine, cysteine, kunitz, aspartic and metallo-proteases and are tested against insect pests using both *in vitro* assays using gut extracts containing proteases and using *in vivo* with artificial diet bioassays (Lawrence and Koundal 2002). Furthermore, transgenic plants expressing serine and cysteine PIs have shown some resistance to Lepidopteran and Coleopteran insect pests (De Leo et al. 2001; Falco and Silva-Filho 2003; Alfonso-Rubi et al. 2003).

PIs have only had limited success, this is mainly due to successful adaptations by insect pests; an extreme adaptation is utilized by Hemipteran insects which feed using a stylet and utilise free amino acids rather than mechanically chew on plant tissue. Other insects rely heavily on salivary and gut proteolysis to digest proteins. They are able to stimulate protease activity, up regulating inhibitor-sensitive proteinases to negate the effects of PIs, within hours of application. Due to these limitations, the immediate future for PIs for use as a sole insecticide remains under question (Gatehouse, 2011).

1.3.2.2 *Bacillus thuringiensis (Bt)* toxins

Commercial genetically modified (GM) insecticides include the *Bacillus thurigensis (*Bt*)* family of toxins which are currently the only major commercially available insect resistant transgenic

crops. Bt are a gram-positive, soil dwelling bacteria with entomopathogenic properties, and were first isolated in 1901 by Sjigetane Ishiwatari and then again by Ernst Berliner in 1915 (Sanajja, et al, 2011). Originally Bt toxins were introduced commercially into cotton to help protect against cotton bollworm, by Monsanto in 1996. With over 75 million hectares of transgenic Bt crop grown in 2013, they are widely successful (James, 2013; Tabashnik et al., 2013).

1.3.2.3 *Bacillus thurigensis* toxin mechanism

There are four non-phylogenetically related protein families of Bt toxins encoded on the Bt megaplasmid, which is over 50,000bp in size. Crystal (Cry) and Cytolitic (Cyt) are classes of pore membrane toxins (δ-endotoxins), synthesized by Bt at the onset of sporulation and during the stationary growth phase. The majority of Bt toxins contain a three domain toxin, crystal protein (Cry) protein (Bravo et al., 2007). The mechanism of action for Cry toxins has been primarily described in Lepidopteran insects: Bt toxins are ingested and are solubilized in the midgut via G- protein mediated apoptosis post receptor binding (Zhang et al.,2006). Bt toxins are subsequently activated by the proteolytic removal of an N terminal peptide by midgut proteases; activated Bt toxins then bind to specific receptors (Zhang et al., 2006). This causes the formation of lytic pores, and cell lysis occurs eventually leading to insect death (Bravo et al., 2007). Furthermore, there are no negative effects of Bt toxins on human, vertebrates or plants which do not possess the high affinity Cry specific receptors (Shimada et al., 2006)

Different Cry toxins have different target sites, and so this class of insecticide can target a wide range of insect species including Lepidoptera, Coleoptera, Hymenoptera and Diptera insects (Bravo et al., 2007). However, it must be noted that some of the most destructive pests including aphids are not affected by the action of Cry toxins. In contrast the Cyt toxins directly interact with membrane lipids and insert into the membrane and are primarily active against dipteran pests (Bravo et al., 2007). A major advantage of all Bt toxins is their specificity towards insect pests as well as being harmless to humans, vertebrates and plants (Federici, 2002).

1.3.3 Genetic improvement

The exogenous application of Bt toxins is ineffective, as rain removes exogenously applied sprays, UV light inactivates certain Cry proteins and pest species are also poorly targeted (Ignoffo et al., 1981). Controlling insect numbers through the use of environmentally friendly pesticides and insect resistance crops is an attractive goal for the scientific community. GM organisms are the most common form of biotechnology employed in modern agriculture, with GM crops grown on 120 million hectares worldwide in 2012 (James, 2012). Meta-analysis has revealed that GM improvement has led to a 22% increase in crop yields since their commercial introduction in the mid-1990s (Klümper and Qaim, 2014).

Commercially available GM insecticidal crops are all currently expressing Bt toxins; with almost all expressing the 3-d domain Cry proteins. However, Bt transgenics are not without their problems, one being the gene sequence encoding 3d Cry protein is from a bacterial source and therefore does not integrate functionally into crop plants unless extensive changes are made.

Despite the successes of Bt toxins as insecticides, resistance to Bt crops was first noted in bollworm *Helicoverpa zea* between 2003 and 2006 (Jackson et al., 2006). Resistance can be seen within just 4-10 generations in the laboratory (Tabasnik et al., 2013); with most of the reported cases of resistance against the Cry1A family of toxins. Hemipteran insects including white flies and aphids which are serious pests are also not affected by Bt toxins (Mellet and Schoeman, 2007). The continued resistance to Bt toxins threatens the efficacy of Bt toxins and highlights the need to develop new insecticides.

The adoption of GM crops for insect resistant and herbicide tolerance has led to a reduction in pesticide spraying by 553 million kg, (8.6%) since 1996 (Brookes and Barfoot, 2015). There is a large market for GM produce especially outside the European Union (EU), however GM crops grown within the EU account for only a tiny percentage of total world production (0.03%) (Brandt, 2003). The EU complex and tight regulations regarding GM crops and many conventional insecticides, means the development of novel non-GM pesticides is vital (2009/128/EC and 91/414/EEC).

1.3.3.2 Gene Pyramiding

More recently an approach known as gene pyramiding, otherwise referred to as gene stacking, has been introduce and is expected to be the most effective way of slowing the evolution of insecticide resistance (Tabashnik et al., 2009). Gene pyramiding involves adding multiple genes and traits into a single plant; transgenic crops incorporating two toxins targeting more than one receptor in an insect reduces the likelihood of that insect developing full resistance against the insecticidal crop.

Pyramiding has been used to develop second generation plants, containing two Bt toxins active against the same pest (Tabashnik et al., 2013). In broccoli plants incorporating both the Cry1Ac and Cry1C Bt genes have controlled diamondback moths resistant to either single protein (Cao et al., 2002). Furthermore, pyramiding of the same genes in crops has been shown to increase the range of toxicity of insecticides (Zhao et al., 2003).

1.3.4 Pest management

Modern agriculture practices that attempt to combat the shortage of food supply have led to the introduction of monocultures of crops and have led to increased opportunities for insect pests to thrive (Andow, 1983).

Good integrated pest management and farming practices can help reduce the risk of crops being lost due to pests with benefits being seen around the world (Lewis et al 1997; Pretty and Bharucha, 2015). Better management of the land has been seen as one of the primary strategies to slow down the development of resistance to insecticides in the field, including resistance to Bt (Wilkins, 2007). The approach in essence creates refuges for insects to feed alongside crop plants and promotes the survival of insects that have not developed resistance to the insecticides present. This allows a percentage of the population to remain susceptible and keep pests below economically threshold levels (Carriere et al., 2003). A number of integrated pest management programmes have been successful particularly across Asia in reducing pesticide use (Pretty and Bharuca, 2015). However as integrated pest management is only one component of a strategic approach against crop pests, it alone is not sufficient to overcome all the limitations of current insecticides.

1.4 Spider Venoms

Spiders evolved from an arachnid ancestor in the carboniferous period over 380 million years ago and have much biological and ecological diversity with over 40,000 described species (Garrison et al., 2016). Furthermore, there is estimated to be over 100,000 spider species that have not yet been classified (Garrison et al., 2016). Spiders are also the most speciose venomous organism alongside the beetle, although not all spiders are harmful or indeed venomous at all (Windley et al., 2012). Many arthropods venoms have evolved to contain a cocktail of chemicals to specifically stun or kill certain types of prey (Liu et al., 2009).

Spider venoms are less well studied than those venoms from other taxa with only approximately 0.4% of the extant species being described (Platnick, 2011). Even a conservative estimate of the peptide diversity in spider venoms leads to an approximate total of 10 million polypeptides (Saez et al., 2010). The venomous cocktails are generally comprised of a heterogeneous mixture of salts, low molecular mass organic molecules (1 kDa), polypeptides (3–6 kDa with three-five disulphide bonds), and a small number of higher molecular mass proteins (approximately 10 kDa) (Escoubas et al., 2000; Rash and Hodgson, 2002).

The exact mechanism as to why there are so many peptides in spider venom remains unknown, however it has been suggested that spiders have developed combinational libraries with various activities (anti-microbial, cytolytic enzymatic and neurotoxic), evolving from ancient polypeptide precursor genes (Windley et al., 2012). These multicomponent venom cocktails are poorly understood, in part due the complexity of the interactions between the various peptides (Saez et al., 2010). Enzymes from saliva and digestive fluids can easily contaminate the venoms when they are ‘milked’ from the spider by electrical stimulation and this sometimes makes it difficult to ascertain the origin of the various components (Escoubas et al., 2000).

1.4.1 Neurotoxins isolated from Australian funnel-web spider (*Hadronyche versuta*)

Nevertheless, spider venoms are rich in neurotoxic peptides that affect ion channels and to a lesser extent neurotransmitter exocytosis (Windley et al., 2012). Most exert effects by interfering with ion channel function via voltage-gated sodium channels whilst others target calcium or potassium channels. Many spider venoms are dominated by small disulfide rich peptides, with an increasing number of peptides derived from spider venom being characterized- and over 800 described on ArachnoServer 2.0 as of 2012. (Windley et al., 2012).

Many of these disulfide rich peptides are now being researched as potential therapeutic agents for humans due to their ability to affect the activity of specific ion channels and potency for particular targets (King, 2011).

Like chemically synthesized pyrethroids and dihydropyrazoles, disulfide rich neurotoxins from spider venoms target sodium voltage gated ion channels in an insect's presynaptic neuron (figure 1.2). Figure 1.2 also shows that disulphide rich toxins also have other sites of action, and these sites are the same as the chemically synthesized insecticides, including the nAChR in the post synaptic neuron.

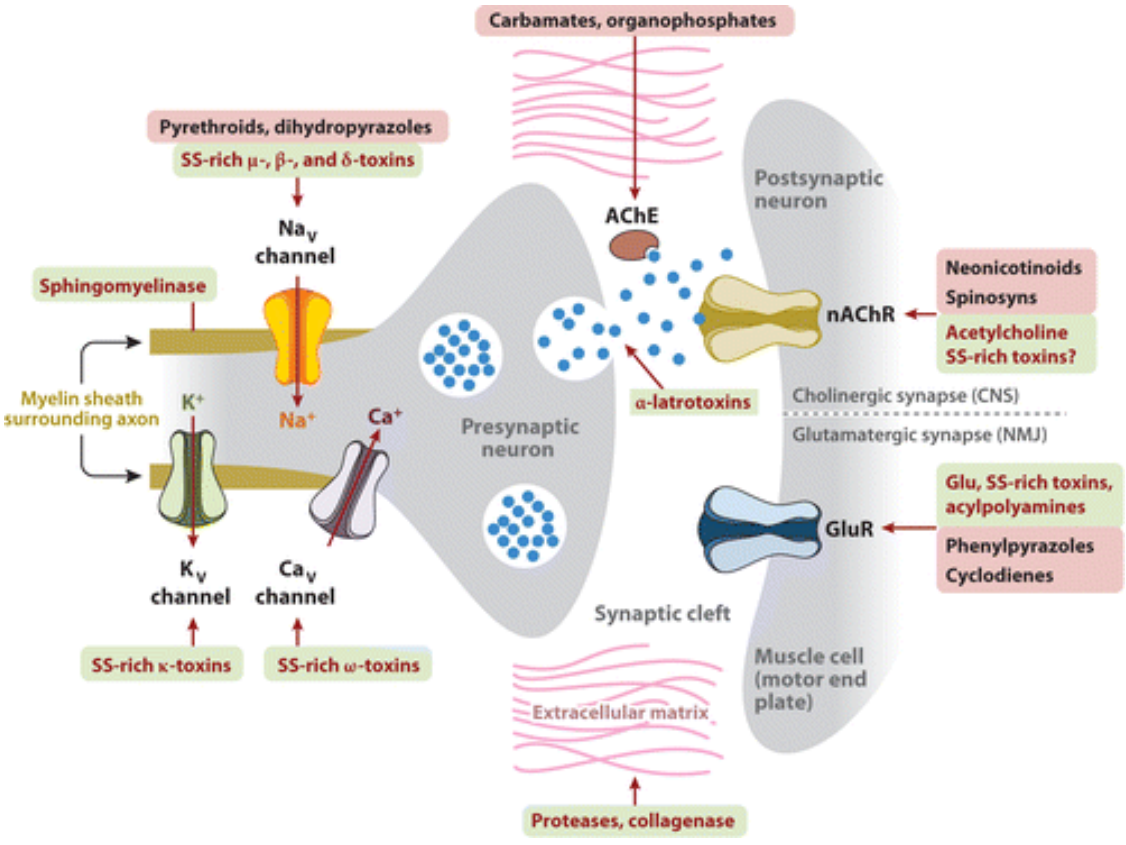

Figure 1.2. Disulphide rich neurotoxins target calcium, potassium and sodium voltage gated ion channels in the presynaptic neuron. Diagram of an insect synapse, molecular targets taken from King GF, Hardy MC, 2013.

Since the ω-atracotoxin-Hv1a (ω-Hv1a-ACTX; Hv1a) peptide was originally isolated from *Hadronyche versuta*, more peptides have been isolated with five peptides been found differing by only 1-3 aa residues (Wang et al, 1999). Table 1.1 shows a large number of peptides from *H. versuta* with similar molecular weights targets these voltage gated channels in insects. More recently ω-atracotoxin-Hv1g (ω-ACTX-Hv1g; Hv1g) has also been isolated from *H. versuta* venom.

Table 1.1 A summary of insecticidal peptides isolated from *Hadronyche versuta* venom and their molecular targets.

Peptide	**Spider source**	**Molecular weight (kDa)**	**Target**
κ-HXTX-Hv1a	*Hadronyche versuta*	3,685	Insect specific CaV (High and Low voltage)
κ-HXTX-Hv1b	*Hadronyche versuta*	3,651	BK_{Ca}
κ-HXTX-Hv1c	*Hadronyche versuta*	3,768	BK_{Ca}
ω-HXTX-Hv1a	*Hadronyche versuta*	4, 055	BK_{Ca}

1.4.2 ω-Hv1a-ACTX peptide isolated from Australian funnel-web spider.

Atkinson et al., 1998, first demonstrated the potency of crude venom from *Hadronyche versuta*, the Australian Blue Mountains funnel web spider towards Lepidopteran larvae. The recently reclassified, ω-Hv1a-ACTX (Hv1a), is a 37-residue long peptide, from *H. versuta* and is the best studied member of ω-atracotoxin family which comprises of peptides between 37-38 residues in length (Wang et al., 1999).

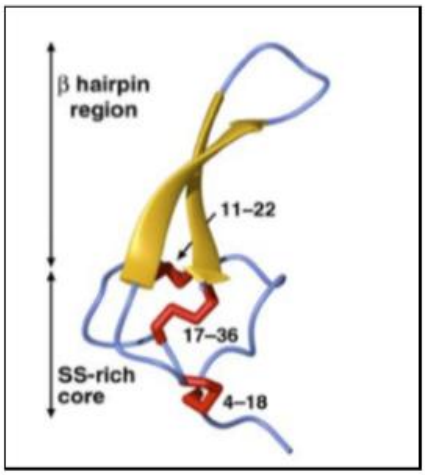

Figure 1.3 Ribbon diagram of Hv1a, depicting the disulphide rich core and beta hair pin domain. The location of three disulphide bonds represented by red-tube. Figure taken from (King, 2007b).

Hv1a has a disordered N-terminus and a disulphide-rich globular core which contains the key residues for insecticidal activity (figure 1.3; figure 1.4) (Tedford et al., 2004). Hv1a is a potent antagonist of voltage gated calcium channels (CaV) specific to insects (Tedford et al., 2001). The ability of Hv1a to target voltage gated calcium channels is not surprising as these channels play a critical role in modulating synaptic transmission in insects (Tedford et al., 2004). Cockroach neuronal membrane assays alongside other insect bioassays identified a spatially contiguous epitope with the amino acid residue Arg35 at the center of this binding flanked by Pro10 and Asn27 which is critical for Hv1a's function (figure 1.4) (Tedford et al., 2004).

Hv1a exhibits high thermal stability and is also highly stable at pH values as low as one or when subjected to organic solvents (Herzig and King, 2015). The remarkable stability of Hv1a which can sustain its structure up to temperatures of 75°C is due to the inhibitor cysteine knot (ICK). Although the ICK motif formed by three intramolecular disulphide bonds is found in many different organisms it is most abundant in spider venom (King and Hardy, 2013). The ICK motif which contains two disulphide bonds, bisected by another disulphide bridge has been demonstrated to be the cause of a high level of resistance to degradation by proteases (Herzig and King, 2015).

The non-lethality of Hv1a to mammals can be demonstrated with doses of the toxin up to 2.5mgkg^{-1} not being able to kill newborn mice (Atkinson et al., 1993). Furthermore, there was no effect of Hv1a when heterologously expressed rat calcium ion channels Cav2.1, CaV2.2 and Cav1.2 in concentrations of Hv1a up to 10μM (Tedford et al., 2004).

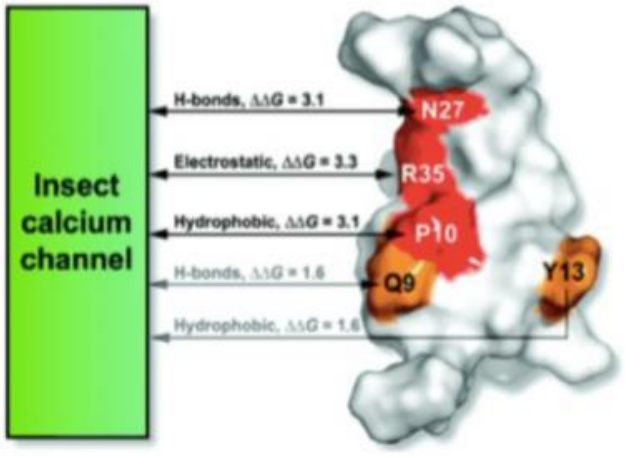

Figure 1.4 3D structure of Hv1a with the major insecticidal sites, Pro10, Asn27, and Arg35 highlighted in red in the conserved B-hairpin domain. Figure taken from Tedford et al., 2004.

Hv1a is highly toxic, by injection, towards many insect pests including those from Lepidopteran, Coleopteran, Dipteran and Ditcyopteran orders (Mukherjee et al., 2006). One

advantage of Hv1a, is that it lacks toxicity to *Apis mellifera* (honeybees) which are major pollinators. There is no significant effect to learning and memory after the Hv1a toxin reaches the brain of adult bees when treated with both acute and chronic doses (King and Hardy, 2013; Nakasu et al., 2014). Furthermore *A. mellifera* larvae were unaffected as they were able to degrade Hv1a-GNA (Nakasu et al., 2014b).

Due to the phyletic specificity of Hv1a and its impressive potency, it is considered to be a highly attractive candidate for further development of a novel insecticide (Fitches et al., 2012; Nakasu et al., 2014). Although highly toxic by injection, Hv1a and other venom peptides are much less ineffective when ingested by insect orders (Fitches et al., 2012). This is in part due to insect gut proteolysis, ineffective delivery to the Central Nervous System (CNS) and therefore the inability of the neurotoxic peptide to reach the site of action in the neuron (Fitches et al., 2012).

1.4.3 ω-Hv1a-Hv1g (Hv1g) isolated from *Hadronyche versuta*.

There is currently no published literature on the spider toxin ω-Hv1a-Hv1g (Hv1g) which has recently been isolated from *Hadronyche versuta.* Its primary amino acid sequence is also identical to the Arg1 toxin isolated from *Atrax robustus* (Wood et al., 2009).

Homological data suggests it is likely to be effective against insect specific calcium ion channels and not mammalian channels.

1.5.1 Plant Lectins

Plant lectins are carbohydrate binding proteins which can be divided into four classes and further classified into 12 families (Macedo et al., 2015). Several hundred lectins have been isolated since their discovery with lectins containing at least one non-catalytic domain that binds reversibly to specific oligo- or monosaccharides (Peumans and Van Damme, 1995). The role of lectins in plant physiology is unclear (Van Damme et al., 1998), they were previously thought primarily to be a nitrogen store, yet have since been shown to play a physiological role in plant defense (Peumans and Van Damme, 1995). Lectins are highly resistant to plant degradation and have been used to produce pesticides (Lam and Ng, 2011). Although their insecticidal activity has received particular attention, lectins have also been studied due to their antifungal and anti-tumour properties (Regente et al., 2014; Shi et al., 2014). Of the currently described plant lectins, *Galanthus nivalis* agglutinin (GNA) has been the most widely studied (Van Damme et al., 1987; Van Damme et al., 1991, Fouquaert et al., 2007, Pusztai, 1991; Poulsen et al., 2007).

1.5.2 *Galanthus nivalis* agglutinin (GNA)

Galanthus nivalis agglutinin (GNA) (figure 1.5) was originally isolated from the bulbs of the snowdrop (*Galanthus nivalis*) (Van Damme et al., 1987). Like many plant lectins, it is synthesized *in planta* as a preproprotein and post translation is cleaved (Van Damme et al.,

1991). This removal of 24 amino acids at the C terminus changes the higher molecular weight precursor from a 15kDa pre-protein into the mature 12.5kDa lectin. This cleavage is sufficient to change the subcellular targeting of the now mature GNA peptide, normally a vascular protein to the nuclear or cytoplasmic compartment (Fouquaert et al., 2007). However, this C terminal pro-peptide does not seem to be involved in lectin function as removal doesn't affect the molecules activity. One property of GNA is that it can be easily purified from snowdrops, as it is stable at a high temperature and over a wide range of pH (Van Damme et al., 1987).

The GNA crystal structure elucidates a novel structure, including a three-fold symmetric beta-sheet polypeptide fold. The three antiparallel four stranded beta sheets, are arranged as a 12 stranded beta barrel. The 50kDa tetrameric protein, composed of 12.5kDa subunits of GNA displays 222 symmetry, it has three orthogonal two-fold axes in the dihedral point group D2. GNA exhibits specificity towards α-1,3 or α-1,6 linked D-mannose oligomers, with the high affinity mannose binding site is located at the dimer interface (Hester et al., 1995).

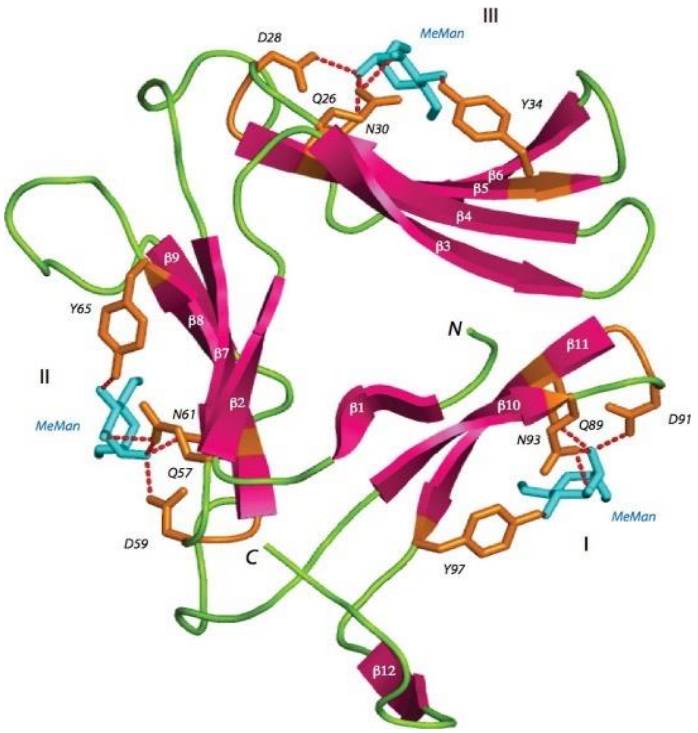

Figure 1.5. A ribbon diagram of GNA, including the pink labelled antiparallel four stranded beta sheets. Not represented are the conserved valine residues required for carbohydrate binding. Figure taken from Van Damme et al., 2007.

GNA, is not glycosylated and is able to agglutinate rabbit erythrocytes (Van Damme et al., 1991) and has shown to be particularly useful due to its slight toxic activity against hemipteran orders (Rao et al., 1998). Additionally, GNA is slightly toxic to multiple tissue feeding insects, including Lepidopteran, Coleopteran and Hemipteran insect pests. (Fitches et al., 1997; Nutt et

al., 1999). Not only does GNA reduce survival and fecundity in insects but also slows development of insect larvae (Gatehouse et al., 1995).

Despite the later controversial episode in 1998 involving scientist Árpád Pusztai, GNA has been shown to be non-toxic to higher mammals (Poulsen et al. 2007). In 1991, Pusztai showed that GNA binds to the epithelial cells, however importantly no effects were found in a long-term feeding experiment of rats with transgenic rice expressing GNA as they lack the mannose receptors required (Pusztai, 1991; Poulsen et al., 2007).

It has been demonstrated that GNA is transported across from the gut into the haemolymph as intact protein after oral ingestion (Fitches and Gatehouse, 1998, Fitches et al., 2001, 2004). GNA has been shown to cross the insect gut in Lepidoptera (Fitches et al., 2001), Coleoptera and Homoptera insects (Hogervorst et al., 2006). The mechanism of GNA's insecticidal activity is still poorly understood however unpublished data by Bonning et al., hypothesized that it reaches the hemolymph at least partly via clatherin receptor mediated endocytosis, with GNA binding to glyco-proteins on the luminal surface of the gut epithelium. This binding is thought to be concentrated in the midgut region and it has been demonstrated that GNA could bind to the gut of the rice brown plant hopper (*Nilaparvata lugens*) (Powell et al., 1998). Recently, using midgut cells in the Cotton Leafworm (*Spodoptera littoralis*) (Walski et al., 2017 have shown that GNA tagged with fluorescein isothiocyanate (FITC) binds preferentially to N-acetylglucosamine (GlcNAc) oligomers in the midgut microvilli. It is hypothesized that the transport of GNA from the gut into fat bodies, ovarioles and haemolymph occurs via clatherin mediated receptor endocytosis (Powell et al., 1998; Fitches et al., 2001, 2004).

1.5.3 Limitations of *Galanthus nivalis* Agglutinin (GNA)

Compared to the highly specific Bt Cry proteins, lectins like GNA have a relatively broad range of activity and therefore more likely to have unintended effects (Gatehouse et al., 1996, Rao et al., 1998, Malone et al., 2008). Some studies have suggested that GNA, has a negative impact on the larvae of *Chrysoperla carnea*, a key insect predator (Li and Romeis, 2009; Lawo and Romeis 2008).

Despite the potential of GNA as an insecticide, and the successful expression of GNA in tobacco, potato, rice and wheat conferring resistance to *N. lugens* and *Mysuz persicae* (Gatehouse et al., 1998, Rao et al., 1998, Hilder et al., 1995, Stoger et al., 1999) insect pests are not able to be controlled by transgenic plants expressing GNA alone. Thus require the use of other insect predators and parasitoids in order to tackle insect pests (Van Emden, 1999).

1.6.1 Recombinant fusion protein technology.

Fusion proteins, produced using recombinant DNA technology, are made up from genes originally encoding for two separate proteins. Recombinant fusion proteins have been used for a

wide number of applications including use as a new class of therapeutic agents. (Beck and Reichert, 2011). They are also promising tools for use as novel insecticides, enhancing toxicity and delaying development of insecticide resistance against insect pests (Fitches et al., 2012).

1.6.2 Hv1a/GNA containing fusion proteins

The fusion protein approach offers a way to enhance the oral insecticidal activity of peptides from spider, and scorpion venoms. Previously it has been shown that the toxicity of venom peptides such as Hv1a can be enhanced when fused to GNA (Fitches et al., 2012). The current hypothesis is that GNA is vital for this enhancement and that GNA mediates the delivery of Hv1a to the site of action. GNA been termed as a "carrier", as the Hv1a/GNA fusion is transported to haemolymph and CNS, is thought to anchor binding of the toxin to nerve tissue and increase the concentration of toxin at the site of action (Fitches et al., 2012). The use of GNA as a carrier was first shown by Fitches et al., (2002) when GNA was fused to the insect neuropeptide 'allatostatin', derived from *Manduca sexta*. Following oral ingestion of this fusion protein when incorporated in the diet at 1.5% *Lacanobia oleracae* larvae displayed reduced growth (Fitches et al, 2002, 2004).

It appears that GNA also helps with correct folding of fusion proteins when they are made recombinantly (Yang et al., 2014). The Hv1a/GNA fusion protein has been shown to have oral activity against Lepidopteran, Coleopteran, Dipteran and Hemipteran insect orders (Fitches et al., 2012; Back, 2011).

1.6.3 Fusion protein production using *Pichia pastoris* as an expression system.

Naturally, the production of recombinant protein insecticides on a large industrial scale will only be cost effective if high expression levels can be reached in host strains from which they are made and purified. *Pichia pastoris*, produces high yields of folded protein and is the expression system used for the production of recombinant fusion protein; it is simple and relatively inexpensive to scale up from a relatively small shake flask culture to a bench top fermenter (Bretthaur and Castellino, 1999; Higgins and Cregg, 1998). *Pichia pastoris* is also as capable of performing post-translational modifications, including disulphide bridge formation which is critical for Hv1as function and N– and O- linked glycosylation (Higgins and Cregg, 1998) Transformed *P. pastoris* secretes recombinant proteins into the culture medium via the α-factor prepro-secretory signal from *Saccharomyces cerevisiae* which is contained within the pGAPzαB expression vector. *P. pastoris* doesn't secrete many of its own proteins naturally, although the culture media contains proteins other than the recombinant protein secreted by the α-factor this is approach keeps these to a minimum (Tschopp et al., 1987).

The Hv1a encoding gene for expression was originally produced from a series of overlapping oligonucleotides, codon optimized for yeast. Purification was enhanced in a single step process by the poly (6) histidine tag, which enables a single-step purification. This reduces the time that

proteins are subject to any proteases present in the culture media, helping minimize any cleavage that may occur.

Proteolysis and cleavage reduces the amount of intact fusion protein that is produced; work was previously carried out to increase efficiency of protein production by Pyati et al. (2014), with the introduction of multiple expression cassettes. The Hv1a/GNA fusion protein is vulnerable to proteolysis during production in the yeast strain, *Pichia pastoris*. The KEX2 gene product is one such protease and is required to cleave at the amino acid sequences N-Arg-Arg/-C and N-Lys-Arg/-C and cleaves the α-factor from the fusion protein during production. Therefore, modifications were made to remove a potential Kex2 cleavage site naturally present at the C terminus of the Hv1a peptide with the 34th lysine being substituted for an arginine (Pyati et al., 2014). This fusion protein variant is referred to as the modified Hv1a peptide (ModHv1a) throughout this thesis.

1.6.4 Expression of Hv1a/GNA fusion proteins *in planta.*

The expression of the snowdrop lectin, GNA in transgenic rice plants has been shown to confer resistance to rice brown planthopper (*Nilaparvata lugens*) (Rao et al., 1998). The gene encoding GNA has also been expressed in wheat (Stoger et al., 1999), papaya (McCafferty et al., 2008), sugarcane (Nutt et al., 1999), tobacco (Hilder et al., 1995) and potato (Gatehouse et al., 1996).

Nakasu et al., (2014) demonstrated that it was possible to express Hv1a-GNA in *Arabidopsis thaliana (*var Columbia) and that these fusion proteins can be delivered to aphids. In detached bio leaf assays of transgenic *A. thaliana* containing the Hv1a-GNA fusion protein, the generalist feeding, peach potato aphid *Myzus persicae,* had 40% mortality, 7 days after ingestion (Nakasu et al., 2014). The work by Nakasu et al. (2014) also showed that the toxicity of Hv1a-GNA combined is greater than the toxicity when GNA is used alone to *M. persicae*.

1.7 Aims and objectives of study

The ultimate aim of this thesis' study is to compare the expression profiles of fusion protein variants. The fusion proteins under investigation have the Hv1a or ModHv1a toxins orientated at the N or the C terminus of the GNA, with a linker region of 3 alanine's between them. The four fusion protein variants, all containing a Histidine tag for purification are from hear on in referred to as His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, ModHv1a-GNA-His. Together with oral bioassays, the insecticidal activity of the four fusion protein variants, is compared. This work will contribute to the selection of the best candidate for fusion protein expression and insecticidal activity in planta.

Transiently expressed fusion protein in a dicot plant will be produced in order to compare the expression profile to a similar fusion protein product purified from transformed yeast. It may

elucidate whether it is sensible to continue the project with the next foreseeable stage which is the generation of stable expressing plant lines, most likely in *Arabidopsis*.

The project will involve:

1) The production and purification of His-GNA-Hv1a in *Pichia pastoris*

2) Comparative expression profile analysis of four different fusion proteins variants, His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, ModHv1a-GNA-His produced in *Pichia pastoris*

3) Assessment of biological activity including insecticidal activity analysis of four different fusion proteins variants, His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, ModHv1a-GNA-His produced in *Pichia pastoris*

4) The production of *Nicotiana benthamiana* transiently expressing the fusion protein GNA-ModHv1a using the Gateway Cloning System.

5) Work will establish whether both toxins are active in a novel fusion protein (ProHvg1-GNA-Hv1a-His; DT1) which incorporates two different spider toxins fused to either terminus of GNA.

Chapter 2.0 Materials and Methods.

2.1 Standard Molecular biology techniques.

2.1.1 Sterilisation techniques.

All necessary procedures were carried out in a sterile environment, achieved either by use of a Monmouth Sterilisation flow hood which had been treated with 70% ethanol spray, or in the close proximity to a Bunsen flame. Where appropriate, media, tips and other apparatus were autoclaved prior to use.

2.1.2 Oligonucleotides

Oligonucleotides were designed, then synthesised and supplied by Integrated DNA technologies (IDT). Oligonucleotides were subsequently re-suspended in nuclease free water (NFW) and made up to a concentration of 100 µM. Primer oligonucleotides were then further diluted to a final concentration of 10 µM when used.

2.1.3 Plasmid DNA Minipreparation

DNA purification was carried out using the alkaline phosphatase lysis method (Birnboim H.C and Doly J, 1979), with the Wizard Plus SV Miniprep DNA purification system provided by Promega. The protocol was followed with the minor modification of allowing the sample rest for 5 minutes after adding 100 µl of NFW in order to elute increased amounts of plasmid DNA from the column.

2.1.4 Polymerase Chain Reactions (PCR)

PCR reactions were carried out at either in 20 µl or 50 µl reactions depending on how much product was required in an Applied Biosystem GeneAmp PCR system 240 machine. High fidelity polymerase, either Phusion polymerase (Thermo) or HIFI polymerase (PCR Biosystems) was used to generate blunt ended PCR products. BioTaq polymerase (Bioline) was used for colony PCR and other screening PCRs, where it was acceptable to leave an overhang.

Initial denaturing conditions were set as 98 °C for three minutes, this was extended for up to 10 minutes for colony PCRs. This was followed by 25 cycles of denaturation (at 98 °C), annealing and extension steps. Annealing temperatures and extension times were modified according to the reaction. After 25 cycles a final 10 minute extension step was carried out.

2.1.5 Agarose gel electrophoresis of DNA

Gel electrophoresis was used to separate DNA based on base pair weight. Gels contained 1% (w/v) or 2% (w/v) agarose with 1X Tris acetate EDTA (TAE) buffer ((made by diluting a 50X TAE stock buffer (2 M Tris-HCl, 2 M Glacial Acetic acid, 50 mM EDTA pH 8.0) and used to

separate the DNA by molecular weight. A 1% agarose gel was always made and used if DNA was to be extracted from the gel.

Powered agar was dissolved in TAE buffer in a microwave and ethidium bromide was added when the agar had cooled. Appropriate volumes of 5 X loading dye (10 mM Tris/HCL pH 8.0, 10 mM EDTA, 20% (w/v) glycerol, 0.1% (v/v) Fast Orange G) was added to all samples before being loaded into the wells. DNA Hyperladder was run alongside PCR samples in order to measure both the molecular weight and amount of DNA in the wells. Gels were run at 100 V and at maximum current, typically for 30 minutes, whilst submersed in 1X TAE buffer. DNA was subsequently visualised under UV light using a Geneflash Syngene UV cabinet. A photo of DNA was subsequently captured and printed for reference.

2.1.6 Extraction and purification of DNA from agarose gels

DNA gel bands were sliced using a clean, sharp, single-sided blade and the DNA was subsequently extracted from the 1% agarose gel slice using the QIAquick Gel extraction kit (Qiagen). The manufacturer's protocol was followed with modifications to include a resting step, where samples were placed under a sterile fume hood until all traces of isopropanol evaporated. An extension to the elution step to maximise the amount of eluted product was also implemented. 50 µl of NFW or for increased concentration of DNA, 30 µl of NFW was used to elute the extracted DNA. In some cases, elution buffer (10 mM Tris-Cl, pH 8.5) was used instead of NFW.

2.1.7 Quantification of purified DNA

Typically 2 µl of DNA sample was used to quantify and analyse the purified DNA on a Thermo Scientific NanoDrop TM 1000 Spectrophotometer. Samples were measured against blank measurements of NFW or elution buffer, depending on how the DNA had been eluted. 1-2 µl of DNA was run on a 1% agarose gel after being mixed with the correct volume of loading dye, and measured against 5 µl of Hyperladder. This method allowed the quality of DNA to be visualised i.e. did the DNA sample run as a non-smeared band.

2.1.8 Chloroform extraction for clean-up of DNA

In order to remove any protein or hydrophilic lipids from DNA samples, an equal volume of chloroform was added. The sample was subsequently vortexed and centrifuged at 13,300 rpm for 3 minutes. The less dense aqueous phase containing DNA was removed using a pipette and stored at -20 °C until use.

2.1.9 Ethanol Precipitation for clean-up of DNA

1/10 (v/v) of 3 mM Sodium Acetate was added to DNA and 3 X volume of 100% ethanol was also added. Reactions were left at -20 °C over the weekend or in some cases overnight. The

solution was then centrifuged at 4 °C for twenty minutes at 16000 G in order to produce a pellet. The liquid was siphoned off and washed twice with 50-100 µl of 75% ethanol before being centrifuged again. The pellet was re-suspended in 10 µl of NFW before being quantified and used for subsequent reactions.

2.1.10 Ligation reactions.

Ligation reactions were performed using T4 ligase and 10 X reaction buffer (Promega/Sigma). Reaction volumes were typically 20 µl and with a typical ratio of 1:1, vector to insert used. Reactions were left at 4 °C overnight to allow for complete ligation.

2.1.11 Restriction endonuclease digestion

Restriction digestion reactions were typically in volumes of 20 or 50 µl and were placed at 37 °C for 2 hours or overnight. The compatibility of restriction enzymes and buffers was checked before use, using the manufacturers' protocol.

2.2 Generation of competent cells

2.2.1 DH5 alpha chemically competent cell line generation

DH5 alpha chemically competent cells were generated for use throughout this project. 10 ml LB broth was inoculated with a single colony and left overnight at 37 °C shaking at 220 rpm. This 10 ml culture was then added into 250 ml of SOB, shaking at 250 rpm at a temperature of 18 °C until the liquid sample had an OD600 reading of 0.6. The culture was then cooled on ice for 10 minutes before being centrifuged at 3000 g at 4 °C for 10 minutes. The supernatant was poured off, and the cell pellet was re-suspended in 80 ml ice cold TB Buffer (15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7), and subsequently kept on ice for 10 minutes. Cells were centrifuged again and re-suspended in 20 ml of ice cold TB buffer. The cells were then kept on ice for a further 10 minutes before being aliquoted into a 15 ml microcentrifuge tube and snap frozen in liquid nitrogen. The cells were transferred to -80 °C for long term storage.

2.2.2 TOP10 electro-competent cells.

Competent One Shot TOP10 electro-competent *E. coli* cells were gifted by P. Pyati (Durham University) in 50 µl aliquots and stored at -80 °C.

2.2.3 *Agrobacterium tumefaciens* (strain Agri GV3101) cells.

Competent *Agrobacterium tumefacians* strain GV3101 were given as a gift from Dr Ari Sananadom (Durham University).

2.3 Transformation of competent cells

2.3.1 Transformation of competent cells TOP10 electro competent cells

One shot TOP10 *E. Coli* competent cells were stored at -80 °C and thawed on ice before use. 50 µl of competent cells were then subsequently used for transformation. 1-2 µl of isolated plasmid DNA or Gateway TOPO reaction mix was added to thawed cells and mixed gently. Cells were then transferred to an electroporation cuvette and subject to electroporation using a BioRad Gene Pulser set at 1.8V.

Cells were electro-shocked, with a transformation coefficient above 4 considered good and one above 3.8 was considered acceptable for transformation of *E.coli* cells. Cells were then supplied 250 µl of recovery low salt Luria –Bertani broth (LSLB) or Super Optimal broth with Catabolite repression (SOC) media and placed at 37 °C on a shaker at 200 rpm to recover and allow the expression of antibiotic resistance genes. The cells were subsequently plated out on LSLB agar (1% (w/v) Trypticase Peptone, 0.5% (w/v) yeast extract, 1% (w/v) Sodium chloride and 1.5% (w/v) agar) plates with specific antibiotic resistance which had been warmed prior to being streaked.

2.3.2 Chemical transformation of cells.

DH5 alpha chemically competent cells were thawed on ice for 10 minutes and until all the ice crystals had disappeared. The cells were mixed gently and 50 µl of cells were pipetted into microcentrifuge tubes on ice. Plasmid DNA (1-5 µl) was added and mixed gently without vortexing. The cells, were subsequently placed on ice for 30 minutes, before being placed in a hot water bath to be heat shocked at 42 °C for 30 seconds. After 30 seconds, they were removed from the water bath and placed on ice for 5 minutes and 950 µl of SOB was added. The cells were then placed at 37 °C for 60 minutes to recover. Cells were then gently mixed without vortexing and spread onto pre-warmed selection plates and left to incubate at 37 °C.

2.3.3 *Agrobacterium tumefaciens* transformation.

50 µl of competent GV3101 Agrobacterium cells stored at -80 °C were left to thaw completely on ice. Cells were then left on ice for 5 minutes, before being snap frozen in liquid nitrogen and placed at 37 °C in a water bath. 1 ml of SOC or LB media was then added. For recovery, the cells were placed at 28 °C, shaking at 200 rpms on an orbital shaker for 2 hours. Cells were subsequently centrifuged at 4000 rpm for 1 minute and re-suspended in 50 µl of SOC or LB Agar, and plated on antibiotic selective plates (rifampicin 50 mg/ml, gentamycin 25 mg/ml and plasmid specific (kanamycin 50 mg/ml)) at 28 °C to grow for 24 hours.

2.3.4 DNA Sequencing and analysis.

DNA sequencing was carried out by Durham DBS genomics centre, Department of Biosciences, University of Durham. Primers used to sequence constructs were either sequence or vector specific. The sequences were viewed and analysed using Serial Cloner 2.6.1 and Chromas Lite 2.1.

2.3.5 Bacterial glycerol stocks

E. coli were grown overnight in LSLB agar broth at 37 °C with antibiotic. The culture was centrifuged at 13,000 rpm at room temperature for 1 minute. The pellet was then re-suspended in 750 µl of LSLB broth with 250 µl 70% glycerol which was transferred to cryovials and stored at -80 °C for future use.

2.3.6 Inoculation

All bacterial cultures were inoculated in a sterile environment, either in close proximity to a Bunsen flame or in the Monmouth Sterilisation flow hood. Autoclaved, sterilised pipette tips were also used.

2.4 Standard biochemical techniques

2.4.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE gels) (0.5 M Tris HCl pH 6.8, Distilled water, 10% SDS, Acrylamide, TEMED, 5% APS) were used to analyse protein as described by Laemmli (1970). All gels contained 17.5% acrylamide unless otherwise stated. 5 X SDS Sample loading buffer with β-mercaptoethanol (BME) added was used with all samples. Proteins were then denatured at 100 °C for 10 minutes.

A current was passed through the gel in order to separate the proteins based on their molecular weight and charge (Shapiro et al., 1967). A voltage of 100V was passed through the gel, whilst the protein samples were running through the stacking gel. The lower percentage of acrylamide, alongside the reduced pH allowed the proteins to ‘stack’, therefore entering the resolving gel at the same time. 150 V was applied as samples passed into the resolving gel and finally 200 V was passed through the gel as the protein samples moved through the remainder of the resolving gel. A Pierce Unstained Protein molecular weight marker (Thermo) was run alongside samples to compare the molecular weights of all the bands.

After electrophoresis, gels were either stained in Coomassie brilliant blue or used for a western blot analysis.

2.4.2 Staining using Coomassie Blue.

The stacking gel was removed and Coomassie Brilliant Blue ((0.04% Coomassie brilliant blue R250, 40% methanol, 7% Glacial acetic acid) added, and was left gently rocking for at least one hour. Destain solution (40% (v/v) methanol, 7% (v/v) glacial acetic acid) was then added till the Coomassie that was not bound by protein was removed from the gel, and visual analysis could be carried out.

Protein quantification was done visually by SDS-PAGE gels. The unknown concentration of protein solution was loaded onto the gel, in various dilution and compared to standard GNA concentrations.

2.4.3 Western Blotting, Semidry method

Samples were run on gel using SDS-PAGE prior to being transferred onto nitrocellulose (Hybond ECL, Amersham) membrane using the semi-dry transfer blotting. 3 mm blotting paper (Whatman), which was soaked in semi-dry transfer buffer (48mM Tris-HCL, 39mM Glycine, 20% (v/v) Methanol) for 30 minutes at room temperature. Any air bubbles were removed with a glass rod. A constant current of 150 mA was passed through the blotter, for one hour. Post blot, gel boundaries were marked on the membrane which was subsequently rinsed in Ponceau S stain (10% (v/v) Glacial acetic acid, 0.5% (w/v) and distilled water), until the molecular weight marker was seen. The membrane was rinsed in distilled water before being washed in 50 ml antisera (1X PB. 0.05% 9 (v/v) Tween-20, 5% (w/v) Milk) for one hour on a shaker. 5 ml of antisera containing 2 µl of the appropriate primary antibody (GNA or His specific) was incubated, either for an hour or overnight at 4 °C with shaking dependent on whether the blot was being completed in one day or a two day protocol. The primary antibody was washed off with 3 washes in antisera, left shaking for 10 minutes each time. 4 µl of secondary antibody was then applied in 10 ml of antisera and left for 1-2 hours at room temperature shaking. Subsequently the membrane was washed twice (1XPBS +0.1% Tween 20) for 5 minutes and 15 minutes.

In a dark room, the membrane was carefully matted dry to remove residual distilled water. The chemiluminescent solution containing 5 ml of Solution A (100mM Tris/HCl pH 8.0, 0.2 mM coumaric acid, 1.25mM luminol in 50ml of distilled water) and 15µl of solution B (10% H_20_2 (30% solution) in distilled water) was mixed in the dark and applied to the membrane for one minute. Subsequently the membrane was blotted completely dry with paper towel and placed in the X-ray cassette. In the dark X-ray film was used and exposed for a number of time points, typically at 30 seconds, 1 minute and touch exposure. The membrane was subsequently developed in an X-ograph Imaging Systems Compact X4 automatic developer.

2.4.4 Periodic Acid-Schiff Blot Staining

Samples were run using SDS-PAGE gels and transferred to the electro –blotter for transfer to a nitrocellulose membrane. Any air bubbles between the layers of gel, membrane and blotting paper were removed with a glass rod. A constant current of 150 mA was then passed through the layers for one hour. Post blot, gel boundaries were marked on the membrane which was subsequently washed in distilled water twice. This stage was particularly important to remove any residual SDS buffer, which is known to give major background interference. The gel was further soaked in solution of 1% periodic acid and 3% acetic acid for thirty minutes. Washing in distilled water for a period of 5 minutes, six times removed the solution. The membrane was then incubated in Schiff reagent for 1hour in the dark. Incubated in solution B (0.1% sodium metabisulphite in 10 mM HCl) for 1hour in the dark. Subsequently the membrane was washed several times with solution D (0.5% (w/v) Sodium metabisulfite in 10mM HCl) for at least 3 hours or overnight. Gels were subsequently stored in solution E (7.5% acetic acid, 5% methanol in distilled water). Visual analysis of the membrane was then carried out (Packer et al., 2002).

2.4.5 Deglycosylation reactions

Protein samples were prepared with a maximum of 200 µg of protein dissolved in 30 µl of NFW. 10 µl of 5 X reaction buffer (250mM sodium phosphate buffer pH 7.0) was added, followed by 2.5 µl of denaturation solution (2% SDS 1M β-mercaptoethanol) and mixed. The samples were then denatured at 100 °C for 5 min in a heat block. Subsequently samples were cooled to room temperature. 2.5 µl of TRITON X-100 detergent (15% solution) was added and mixed gently. 1 µl of each enzyme needed for N-linked or O-linked de-glycosylation were then added. N glycosidase F was used for N-linked glycosylation. Endo-α-N-acetylgalactosaminindae, α2-3,6,8,9-Neauraminidase, β1,4-Galactosidase, β-n-Acetylglucsaminidase, Bovine Fetuin were all used in an attempt to deglycosylate O-linked glycans. Finally, the samples were incubated for 72 hours at 37 °C before analysis by SDS-PAGE gel with CBB staining.

2.4.6 *In vitro* haemagglutination of recombinant proteins

Rabbit blood cells in Alsevier's reagent were acquired from TCS Biosciences LTD. The blood cells were washed by the addition of 1 ml of 1 X PBS, and span for 1 minute at 13,400 rpm in a bench top centrifuge. The supernatant was discarded, and the pellet resuspended in 1 ml of 1 X PBS to form the 2% erythrocyte suspension.

The assay was carried out in a round U bottom clear microtitre plate (96 wells). Where 50 µl of 1X PBS was added to each well. 50 µl of a 0.1 mg/ml solution of lectin and 50 µl of erythocyte suspension was then added. A multichannel pipette was used to gently mix and serially dilute the samples.

The assay was left, undisturbed for a minimum of two hours, at room temperature before results were analysed visually. Positive agglutination formed a hazy red well, caused by cross-linked cells.

2.4.7 Bradford assay

The quantity of total protein recovered after plant protein extraction was analysed using a Bradford assay against BSA standards. 5 µl of protein of unknown concentrations was suspended in 20 mM sodium phosphate buffer pH 7.4 (SPB) in a flat bottomed microtitre plate well. Bradford reagent was then added and mixed. Any bubbles were removed by flaming and left for 10 minutes at room temperature. Absorbance was measured at 595 nm wavelength using VeRA max microplate reader. Known BSA concentrations were compared to standard curve ranging from 0.125-1mg/ml. The absorbance was corrected using a blank well of 5µl of SPB and 100µl of Bradford reagent. The concentration of unknown protein was taken by interpolation using a standard curve. All results were carried out in triplicate.

2.5 Production of recombinant insecticidal proteins in *Pichia pastoris*

2.5.1 Bench-top fermentation

A *P.pastoris* clone containing the fusion protein construct was streaked onto a Yeast Extract Peptone-Glycerol (YPG), (2% (w/v) Trypticase Peptone, 1% (w/v) Yeast Extract, 4% (v/v) glycerol and 2% (w/v) agar), with $mgml^{-1}$ zeocin. The plate was then incubated at 30 °C overnight and used to inoculate 100 ml YPG cultures. These cultures were grown for three days, at 30 °C, left shaking at 200 rpm on an orbital shaker. The starter cultures were then used to inoculate 2.5 litres of sterile basal salt medium (26.7 ml/L 85% phosphoric acid, 0.93 g/L calcium sulphate, 18.2 g/L potassium sulphate, 14.9 g/L magnesium sulphate 7-hydrate, 4.13 g/L potassium hydroxide and 40 g/L glycerol) with 13 ml PTM1 salts (6 g/L cupric sulphate 5-hydrate, 0.08g/L sodium iodide, 3g/L manganese sulphate hydrate, 0.2g/L sodium molybdate 2-hydrate, 0.02g/L boric acid, 0.5g/L cobalt chloride, 20g/L zinc chloride, 65g/L iron (II) sulphate 7-hydrate, 0.2g/L blotin and 5ml/L sulphuric acid) in a 7.5L capacity BioFlo 110 bench-top fermentation vessel (New Brunswick Scientific). Fermentation was conducted for three days at 25°C, pH 5.0, 30% dissolved oxygen, continuous agitation, and with a glycerol feed ((50% v/v) glycerol, 30ml antifoam, 12ml PTM1 salts) which were increased from 5-10ml/h over this time period.

2.5.2 Recovery of Recombinant fusion protein

This presence of the alpha (α) factor in pGAPZαB allowed the secretion of the protein expressed by *P. pastoris*. Centrifugation was used to recover the secreted protein present in the media. A Beckman Coulter centrifuge and a J-lite 10500 rotor was used at a speed of 9000 g at 4°C for 30 minutes. The supernatant was subsequently filtered with membrane filters with pores

at 2.7 µM (GFD and GFF; Whatmann) using a vacuum manifold. The supernatant was then loaded onto the HisTrap nickel columns (GE Healthcare) for purification or frozen at -20 °C to be stored for future use.

2.5.3 Recombinant protein purification by immobilised nickel affinity chromatography

Nickel affinity hydrophobic interaction chromatography using HisTrap nickel columns (GE Healthcare) were used to purify the protein. Prior to loading supernatant, it was equilibrated in 4X binding buffer (BB) (50mM sodium acetate, 0.5 NaCl (pH4.0)) to a final concentration of 1XBB and then filtered through 0.7 µM glass microfiber filter using a vacuum manifold. The HisTrap nickel columns were equilibrated using ten times column volume, of 1X binding buffer (1XBB). The first 5 ml of each wash was collected in order to be analysed. Supernatant was kept submerged in ice and loaded onto the columns overnight with a flow rate of 5 $mlmin^{-1}$. After this first loading and subsequent elution, the supernatant flow through was loaded for a second time.

Ten times column volume of 1XBB plus 10 mM Imidazole (0.0 2M sodium phosphate, 10 mM imidazole, 0.4 NaCl (pH7.4)) was passed through the columns in order to remove any weakly bound protein. 1XBB plus 200 mM Imidazole (0.02M sodium phosphate, 300 mM imidazole, 0.4M NaCl (pH 7.4)) was then used to elute the protein from the columns. Flow through from the columns was collected and stored at -20 °C.

Samples of flow through and elution fractions were analysed by SDS-PAGE electrophoresis with CBB staining. The HisTrap nickel columns were stripped and re-charged after every 4-6 loads: they were stripped with buffer (20 mM sodium phosphate, 500 mM Nacl and 50 mM EDTA (pH7.4)) and recharged with 0.1 M $NiSO_4$ to ensure a high recovery. The columns were subsequently stored in 20% ethanol.

2.5.4 Dialysis of Samples.

Elution fractions were pooled and dialysed against distilled water for approximately 24 hours at 4 °C. The distilled water was changed four times over the 24 hour period. The dialysing tubing, with a molecular weight cut off of 12-14 kDa was prepared by cutting it to length and boiling for 10 minutes, in NFW containing 5 mM ammonium hydrogen carbonate and a small amount of EDTA.

2.5.5 Freeze drying of recombinant protein

Dialysed protein samples, were then transferred to a freeze drying flask. Liquid nitrogen was used, and the flask swirled to freeze an even sample against the walls of the flask. The flasks were then put onto a vacuum pump (Christ LOC-IM alpha 1-4) overnight until the protein samples had fully lyophilized. Samples were removed, and the lyophilized powder was subsequently stored at 4°C.

2.6 Insect studies

2.6.1 *Acyrthosiphon pisum* (Pea aphid) cultures

Acyrthosiphon pisum (Pea aphids) were housed in bug dorms on green bean plants in a growth room with constant light at 20°C and 70% relative humidity. A liquid aphid diet was prepared from stock solutions. Stock solutions ((150 mM) (Alanine 50.8 mg , Arginine 300.2 mg, Asparagine 213.9 mg , Aspartate/Aspartic acid 189.7 mg, Cysteine 42.5 mg, Glutamic acid 123.6 mg, Glutamine 241.1 mg, Glycine 9.0 mg, Proline 65.6 mg, Serine 59.9 mg, Tyrosine 10.9 mg , Histidine 182.4 mg, Isoleucine 114.1 mg, Leucine 114.1 mg , Lysine 158.9 mg , Methionine 42.5mg , Phenylalanine 47.1 mg , Threonine 103.6 mg , Tryptophan 58.2 mg , Valine 101.9 mg) dissolved in 50 ml distilled water) were stored at -20 °C until use and prepared as descried in Kunkel (1976) and Prosser and Douglas (1992).

2.6.2 Pea aphid bioassays

Adult *A. pisum* aphids were taken from the cultured plants and placed on artificial diet overnight in order to make sure the nymphs produced and collected the following day were 1 day old. Nymphs produced by these adult aphids were then used for the bioassay. Fifteen nymphs were placed in a small chamber made up of Perspex (figure 2.1) and were trapped by two thin layers of parafilm in between which contained 25 µl of liquid diet mixed with the correct dose of fusion protein; SPB (20 mM NaPO4 with deionised water) was used to make up the rest of the solution. . In the control group fifteen nymphs were treated the same but were not fed fusion protein, and the 25µl solution was made up with liquid diet and SPB.

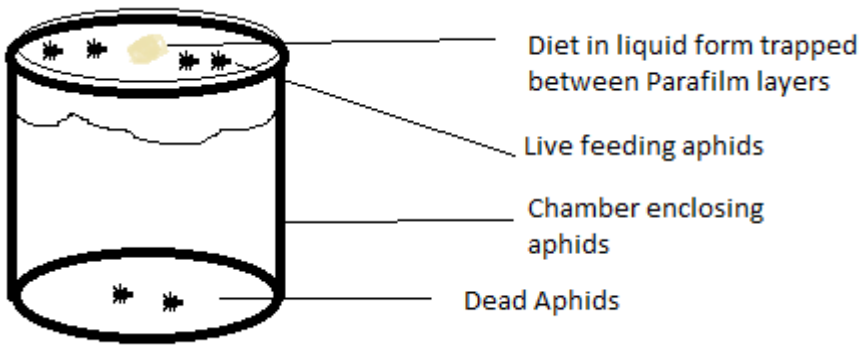

Figure 2.1. *Diagram of an aphid feeding chamber, used in the feeding bioassays*

The diet was renewed every 2-3 days to prevent it going off, with insects transferred to new chambers using the ends of a paint brush. The number of aphids, both dead and alive was counted daily at a similar time and recorded.

2.6.3 Stability of Fusion Protein in aphid diet.

Diet containing the fusion protein was left in the growth room with constant light at 20 °C and 70% relative humidity. 15 day 5 Pea aphids were left to feed on each sample of aphid diet containing fusion protein in aphid chambers for 24, 48 and 72 hours. 15 µl of diet was then removed and analysed on western blot by His-specific antibodies.

2.6.4 Oral ingestion of Fusion Protein

15 aphids were fed on diet containing the correct treatment for time periods of 24, 48 and 72 hours. 10 aphids were then snap frozen in liquid nitrogen and ground up with SPB buffer. Samples were centrifuged at 13,000 rpm at 4 °C for 20 minutes, 10 and 20 µl was then analysed on a Western Blot with GNA specific antibodies

2.6.5 *Mamestra brassicae* cultures

M. brassicae were originally obtained from The Food and Environment Research Agency (FERA Science Ltd.), York and reared at Durham University. The culture was maintained at 25 °C, with a dark: light cycle of 16:4 hours. Artificial diet comprised: (13.3 g bacto-agar, 3.57 g ascorbic acid, 1.77 g sorbic acid, 2.93 g methyl-4-hyroxybenzoate 6.33 g Vitmix (Vanderzant modification vitamin mixture for insects, ICN Biomedicals Ltd, Thame, Oxon., U.K.), 0.123 g ampicillin or 1.00g aureomycin (Cyanamid Ltd, Gosport, Hants., U.K.), 3.67 ml formaldehyde solution (37%), 6.67 g Wesson salts (salt mix W, ICN Biomedicals Ltd.), 74.0 g haricot bean meal, 59.0 g wheat germ, 30.0 g soyabean meal, 20.0 g casein, 33.3 g yeast and distilled water to 1000 ml) as originally described in Bown et al., (1997).

2.6.6 *Mamestra brassicae* injections

M. brassicae, at the fifth instar moulting stage were identified from the culture and used for injection. Controls had a mean weight of 45.2 ± 7.5 mg and were injected with 4 µl of filter sterilized SPB, using a 10 µl Hamilton syringe. Prior to injection, larvae were gassed with carbon dioxide, was used to anesthetize individuals.

Post injection, larvae were left in plastic chamber pots with air vents, with continuous access to artificial diet. Survival of *M. brassicae* larvae was checked and recorded every 24 hours, for 96 hours (4 days).

Protein stock solution was analysed by SDS-PAGE and visualised by CBB staining in order to ensure comparable doses were present in the solution to be injected.

2.6.7 Statistical analysis

Results were collated on Prism 5.0 software (GraphPad Software Inc.) and LC50 values generated. Statistical analysis of Kaplan–Meier insect survival curves were compared using Mantel–Cox log-rank tests, with the accepted level of significance was $P < 0.05$ in all cases.

2.7 Generation of transgenic plants

2.7.1 Gene sequence synthesis

A GNA Leader protein sequence required for expression of GNA in-planta was synthesised from Shiang Gene in China based on the sequence in Blast software.

2.7.2 Fusion Protein Oligonucleotides

Oligonucleotide primer sequences were synthesized by Integrated DNA Technologies for use in PCR amplification and creation of constructs for use in cloning. In order to enter the GATEWAY ™ (Invitrogen) each GNA leader construct contained the CACC amino acid sequence.

GNA Leader forward primer. 5'- CACCATGGCTAAGGCTTCTCTT-3' (22bp, Tm: 54.8)
GNA Leader reverse primer, containing a SalI recognition site and TAT at the C terminus.
5'-TATGTCGACTCCAAGACAAGAAGGAGA (27bp, Tm: 58.2)

All previously made constructs used for templates were supplied as glycerol stocks by Dr P Pyati.
Forward Primers:
GNA-ModHv1a/Hv1a: 5'-ATAGTCGACGACAATATTTTGTAC (24bp, Tm:51)
ModHv1a-GNA: 5'-ATAGTCGACTCTCCAACTTGTATT (24bp, Tm: 52.3)
Hv1a-GNA: 5'-ATAGTCGACTCTCCAACTTGTATT (24bp, Tm: 52.3)

Reverse Primers were generated to meet the requirements of using an N terminal tag for the destination vector pEarlygate201 and contained C terminal stop codons.
GNA-ModHv1a: 5'-CTAATCACATCTTTGAACAGTATTACC
GNA-Hv1a: 5'-CTAATCACATCTTTTAACAGTATTACC
ModHv1a/Hv1a-GNA 5'- CTATCCAGTGGCCCAACGA

2.7.3 Gateway™ Cloning

pENTR, D-TOPO cloning reactions were carried out using 1 µl of vector, 1 µl of DNA at a concentration of approximately 100 ngµl^{-1}.

Positive colonies were analysed by colony PCR with forward M13 primers and gene specific reverse primers. Positive colonies were then sent to DBS genomics for sequencing.

Restriction digestion reactions of the positive pENTR vectors with the enzyme BspH1 in CutSmart buffer were used to cut out the kanamycin resistance gene and the plasmid re-circularised, gel extraction for use in subsequent LR Clonase reactions.

The LR clonase reaction of the GATEWAY™ cloning system was used to produce a destination vector with the correct DNA insert. The destination vector pEarlyGate 201 vector (obtained from Dr A Sanandom) which incorporated a HA tag on the N terminus of the protein was used.

The LR reaction was carried out as of manufacturer's protocol, using 150 ng of pEarleyGate 201 plasmid and 150 ng of pENTR insert. Analysis of positive clones was carried out via colony PCR, and an analytical restriction digest using BspH1 and Sal1.

2.7.4 Plant material and growth conditions.

Nicotiana benthamiana plants were grown by John Simpson (Durham University) in growth rooms until just prior to flowering, when the leaves were of adequate size to be used for agro-infilitration.

2.6.5 Agro-infiltration.

1 ml of DMSO with 0.034 g of acetosyringone was made fresh on the day of use and added to the culture in a concentration of 1000:1 of culture: acetosyringone/DMSO. The new cultures were incubated for 1 hour and 30 minutes before agroinfiltration into three of the oldest leaves of *Nicotiana benthamiana*

Leaves were marked for agro-infiltration with a pen, primarily using the oldest leaves lowest down the stem. Leaves were infiltrated using a 15ml syringe at various points in order to ensure the whole of the leaf was infiltrated. Post infiltration plants were left, to transiently express the protein in temperature and light controlled growth rooms. Protein extraction took place after either 4 days post agroinfiltration.

2.7.6 Protein extraction from *N. benthamiana*.

Infiltrated leaf tissue was snap frozen using liquid nitrogen and ground using a pestle mortar. A teaspoon of polyvinylpolypyrrolidone per gram of leaf of tissue was then added and ground further with extraction buffer (NaCl 150mM, Tris-HCl 100mM (pH7.8), Glycerol 5%, DTT 10mM, EDTA 5mM, TritonX-100 0.1%, Protease Inhibitor cocktail) added and the final concentration made up with SDW. The protein extracted was analysed by Western Blot with GNA specific antibodies.

2.7.7 Estimation of leaf protein concentration by Bradford assay

5 µl of sample, 5 µl of SPB and 100 µl of Bradford reagent were added to each well of a mirotitre plate and the binding of protein molecules to Coomassie dye under acidic conditions resulted in a colour change from brown to blue (Bradford, 1976). Any bubbles in the wells of the plate were removed by a flame and the samples were left to stand for 10 minutes at room temperature. Light absorbance of BCA samples was measured using a 595nm wavelength using VERA max microplate reader. This generated a standard curve using BCA samples with an R^2 value >0.98. Then the light absorbance of all protein samples were measured in triplicates.

Chapter 3: Production and purification of recombinant fusion protein His-GNA-Hv1a in *Pichia pastoris*. The optimisation of intact fusion protein recovery.

3.1 Introduction

Pichia pastoris, is a methylotrophic yeast strain and a proven expression system for a diverse range of recombinant proteins (Kurtzman, 2009; Fitches et al., 2004, 2012). A rapid growth rate, the lack of known human pathogenicity, and ease of genetic manipulation makes *P. pastoris* a useful expression system for recombinant proteins (Lie et al., 2007). *P. pastoris* secretes correctly folded protein and has previously been used to produce recombinant fusion proteins containing atracotoxins (Ahmed, 2014; Fitches et al., 2012). Unlike *Escherichia coli*, *P. pastoris* is able to produce proteins that contain multiple disulphide bridges that are critical to the function of the ω-Hv1a-ACTX (Hv1a) peptide (Cereghino and Cregg, 2000; King et al., 2002)).

Hv1a is a 37aa peptide derived from the Australian Blue Mountains Funnel web spider, *Hadronyche versuta*. *Galanthus nivalis* agglutinin (GNA), from the common snowdrop has been shown to be able to cross the insect gut membrane (Fitches et al., 2004). In GNA/Hv1a fusion proteins GNA acts as a 'carrier' protein mediating Hv1a's delivery to the insect's haemolymph, with these fusion proteins proven to be toxic towards insects after both injection and oral ingestion (Fitches et al., 2012, Pyati et al., 2014). Here, *P. pastoris* was the expression host for the His-GNA-Hv1a construct, one of four GNA/Hv1a fusion protein variants to be described in this thesis.

The expression vector pGAPzαB is propagated in *E. coli*, and contains the alpha factor pre-pro sequence from *Saccharomyces cerevisiae.* This causes fusion protein generated in *P. pastoris* to be secreted into the culture media allowing for simple down-stream purification. Despite this, fusion proteins are still subject to proteolytic cleavage as a result of a number of proteases in *P. pastoris*. The KEX2 gene product is one such protease, however it is required to cleave the α-factor from the fusion protein. The fusion protein construct His-GNA-Hv1a contains a poly-Histidine (6 amino acid) tag, which is present on the N terminus and enables affinity purification of the recombinant protein (Fitches et al., 2012). Immobilised Nickel- affinity chromatography, makes use of histidine's ability to bind chelated transition metal ions and is used to purify the protein. This purification method limits the time fusion proteins are exposed to proteases which could cause the protein to become non-functional (Pyati et al., 2014). Intact fusion protein can then be eluted from Nickel columns to which the protein was bound to, by applying a buffer containing a high concentration of imidazole.

This chapter reports on the fermentation of *P. pastoris* to produce His-GNA-Hv1a. Intact fusion protein from the lyophilised protein powder generated, was to be subsequently used for expression analysis, and biological activity assays against insects. Successful production of large amounts of intact His-GNA-Hv1a is critical for research in this thesis but also for this fusion protein to be a viable commercialised product.

3.2.1 Construct

The His-GNA-Hv1a fusion protein construct (Figure 3.1) used for fermentation in *P. pastoris* had previously been generated by Dr P Pyati. The pre-pro-peptide alpha factor, is cleaved by the KEX2 gene product during fusion protein expression (Pyati et al., 2014). The six histidine residues of 1.06 kDa are positioned at the N terminus, with GNA of 12.09 kDa bound to the N termnius Hv1a (4.06Kda) by a triple alanine (AAA) linker. The molecular weight of the fusion protein is expected to be 17.21 kDa.

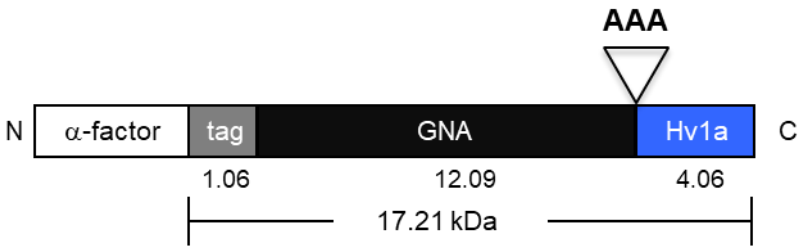

Figure 3.1. Diagrammatic representation of the fusion protein construct His-GNA-Hv1a. The alpha factor (white) on the N terminus is cleaved during expression. GNA (black) is linked to the toxin Hv1a (blue) by a triple alanine amino acid linker region.

3.2.2 Fermentation and expression of His-GNA-Hv1a fusion protein

Bench top fermentation of His-GNA-Hv1a was carried out using an Applikon Bioreactor (Fitches, 2012, Pyati 2014). Three 100 ml yeast extract peptone-glycerol (YPG) shake flasks were inoculated with a yeast strain expressing His-GNA-Hv1a and grown at 30 °C for three days, constantly shaking at 220 rpm. The cultures were subsequently added to the 3 L media in the Applikon Bioreactor and fermentation was run for three days at 30°C, pH 5.0, with 30% dissolved oxygen, continuous agitation, and a glycerol feed which was increased from 5-10 ml/h over this time period. The secretory alpha factor meant that His-GNA-Hv1a was continually being secreted into the media.

During fermenter runs #78 of His-GNA-Hv1a it was noted that the *P. pastoris* culture was not growing as well, compared to previous fermentation run. However, all parameters including the temperature, dissolved oxygen levels and pH remained within range. Poor expression of His-GNA-Hv1a could be seen visually on the day of harvest, with a reduced wet cell pellet weight, compared to previous fermenter runs of His-GNA-Hv1a (data not shown). The expression level of His-GNA-Hv1a fermenter runs was visually quantified using western blot analysis of the neat

culture supernatant, against GNA standards (table 3.1). For fermenter run #78, 10 µl of neat culture supernatant corresponded with 200 ng of recombinant GNA and for fermenter run 50.

Table 3.1. The expression level of calculated, as described above by visual analysis of Western His-GNA-Hv1a fermenter runs #78 and #80.

Fermenter run	Expression Level (mg/L)
#78	20
#80	50

3.2.3 Purification of His-GNA-Hv1a from culture supernatant.

The standard procedure (as described in Chapter 2.5) for purification of recombinant fusion proteins from culture supernatant was initially followed for fermenter runs #77 and 78. 4X Binding Buffer (pH 7.4), was added to neat culture supernatant to make it up to a final concentration of 1XBB. This solution was then filtered using a 2.7 µM filter (Whatmann) and loaded onto His-trap Nickel columns for a one step purification method. After the supernatant (submerged in ice) was loaded onto the columns overnight, a first wash with 1XBB removed any non-specifically bound protein. A 1XBB with 10 mM Imidazole wash, was used to remove any protein which was weakly bound to the purification column. The 200 mM Imidazole elution fractions were recorded on a UV optical chart recorder and peak fractions collected. In comparison to an expected standard purification profile (Figure 3.2 a) where the 10 mM peak is small relative to the 200mM peak, the purification profile peak obtained when supernatant from fermenter run #78 was loaded was much reduced (Figure 3.2 b). The reduction in amplitude of the 200 mM imidazole peak, reflected the reduced amount of protein eluted. (Figure 3.2 b).

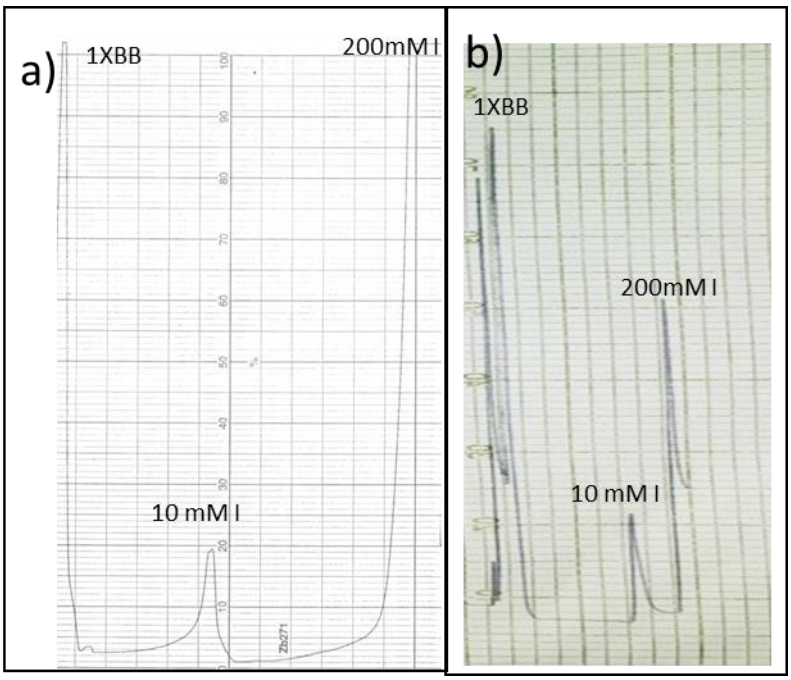

Figure 3.2. a) A standard purification profile generated by the UV optical chart recorder. Wash with 1X Binding buffer (1XBB), the smaller peak shows the 10mM Imidazole peak (10mM I) and 1XBB with 200mM Imidazole peak (200mM I) which is collected. B): The purification profile of His-GNA-Hv1a from fermenter run #78 showing the reduced 1XBB peak, 10mM I and 200mM I peaks.

Post purification elution fractions from 200 mM Imidazole washes were pooled together and dialysed against NFW using 12-14 kDa molecular weight dialysis tubing (GE Healthcare). Dialysis therefore removed any cleaved fusion protein below 12-14 kDa, pigmented components, salts or residual imidazole (which is toxic to insects). Post dialysis, the samples were snap frozen in liquid-nitrogen and then freeze dried until completely lyophilised. This powder was weighed, with only 10 and 18.7 mg of powder for fermenter run #77 and #78 produced respectively.

Lyophilised powder generated from fermenter runs was resuspended at a concentration of 5 $mgml^{-1}$ in 20 mM sodium phosphate buffer at pH 7.4 (SPB). Estimation of intact fusion protein was made by visual analysis of an SDS-PAGE gel stained with Coomassie Blue (CBB). The reasoning behind using a visual estimate and the alternatives quantification methods are discussed in detail in the general discussion of this thesis (Chapter 8). The intensity of stained fusion protein bands was compared with the intensity of staining of known amounts of recombinant GNA standards (Figure 3.3). Purified His-GNA-Hv1a runs as two tightly spaced bands at two different molecular weights, 18.4 kDa and 20 kDa respectively on a 17.5% acrylamide gel (Figure 3.3). Only protein in the higher molecular band was considered as intact fusion protein, based on Q-Star data referred to in Chapter 4 of this thesis, the second band is hypothesised to be cleaved fusion protein, and therefore without the GNA carrier is unlikely to

affect toxicity as is not able to pass through the insect gut intact. Lyophilised powder from fermenter run #77 was quantified to 10% (w/w) on the basis that 20 µg of re-suspended powder visually corresponding to 2 µg of a GNA standards, with 10 and 50 µg of powder relating to 1 and 5µg of GNA, respectively (Figure 3.3 a). Whilst lyophilised powder for #78 was visually quantified to be 25%, on the basis that 2 µg of GNA standard corresponded to 10 µg of resuspended protein powder (Figure 3.3 b).

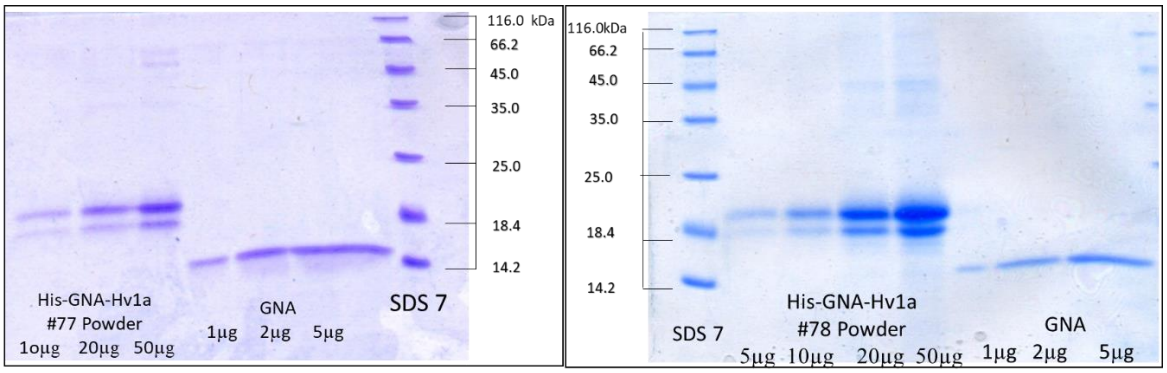

Figure 3.3. SDS-PAGE analysis of protein content in lyophilised samples derived from fermenter runs #77 (a) and #78 (b). Lyophilised powder was resuspended at 5mgml^{-1} before being loaded. GNA standards of 1, 2 and 5µg were loaded for visual quantification of fusion protein. 10% of the powder from run #77 was estimated to be intact His-GNA-HV1a. 25% of the powder from fermenter run #78 contained 25% intact His-GNA-Hv1a..

It was hypothesised that the poor recovery of His-GNA-Hv1a from both fermenter runs was due to poor binding of protein to the His-Trap purification columns which is made possible by the 6 histidine residue tag on the N terminus of the His-GNA-Hv1a protein. Therefore, a western blot using His-specific antibodies was carried out in order to establish if the His tag was lost, by cleavage by *P. pastoris* proteases during fermentation or during dialysis.

A western blot with His specific antibodies (figure 3.4) gave convincing evidence that the His tag was not lost during expression or purification, as the chemiluminescent bands produced were of the weight and pattern expected based on previous Western blots and SDS-page gel electrophoresis of His-GNA-Hv1a. The majority of the intact fusion protein is seen in the 200mM Imidazole wash. However, there was a high percentage of intact fusion protein still present in the supernatant samples after being loaded on the His-Trap Nickel columns twice. This suggests that protein binding to the His-Trap columns was inefficient.

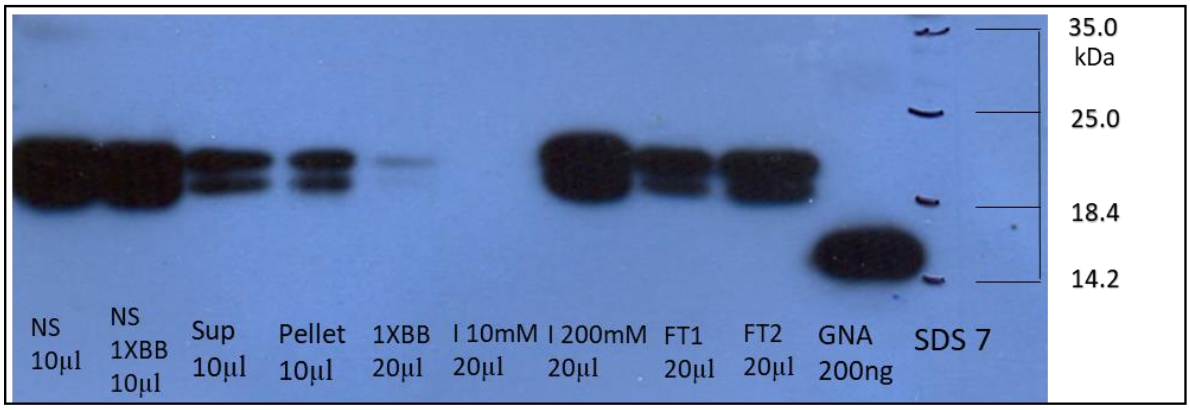

Figure 3.4. His-specific Western Blot of the elution profile of His-GNA-Hv1a from fermenter run #78. Samples were all 20µl unless otherwise stated. 10 µl of neat supernatant (NS), neat supernatant plus 1X binding buffer (NS+BB), 10 µl of supernatant post centrifugation (Sup) 20µl of pellet re-suspended in SPB, the 1XBB wash, and 10mM and 200mM Imidazole elution fraction and supernatant after flow through, alongside a 200 ng GNA standard.

3.2.4. Pre-purification Buffer exchange to raise culture pH

The pH of the neat culture supernatant of fermenter run #80 was measured using a pH probe and litmus paper and was shown to be weakly acidic at a pH 4.75. At pH 4, histidine becomes pronated and is competed off of the Nickel metal ion during purification. Binding buffer washes are made up to pH 7.4 which increases the pH of the supernatant and aids the binding of intact fusion protein to the columns. However, after the addition of 4XBB to 500 ml of culture supernatant to make a final concentration of 1XBB, the pH of the culture supernatant was only raised to pH 5.5.

To enable efficient binding of protein onto the His trap- Nickel column and to increase the amount of protein recovered, the pH of the culture supernatant needs be further increased. Raising the pH by adding 2XBB of pH 8 to 500 ml of supernatant directly, resulted in almost instant, heavy precipitation (data not shown). This precipitation was the result of the fusion protein coming out of solution, as the sudden increase in pH took the recombinant protein past its isoelectric point. The now charged proteins caused it to unfold severely and precipitate rendering it unusable.

Slow buffer exchange of culture supernatant was employed and allowed the pH to be raised without causing precipitation. Neat culture supernatant was filtered, and then dialysed against 1XBB (pH 8) for 2-3 hours at a time, with 3 changes. Slow buffer exchange increased the pH of the supernatant from pH 4.75 to pH 6.96 for supernatant in fermenter run #80 and meant the amount of lyophilised powder recovered from affinity purification to 34 mg. SDS-PAGE gel analysis shows that the use of slow buffer exchange results in no fusion protein left in the supernatant after the first elution with 200 mM Imidazole, and only a minimal amount of protein lost in the 10 mM Imidazole wash fraction (figure 3.5).

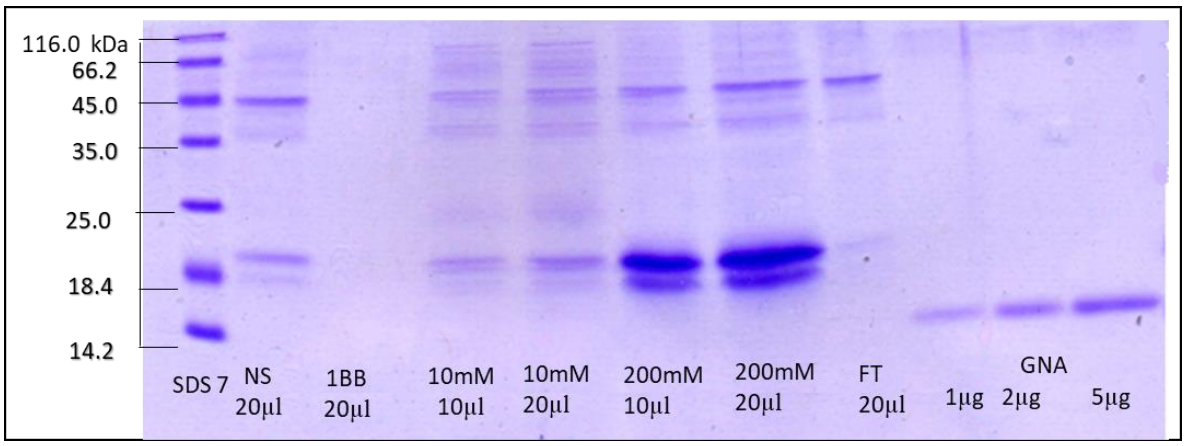

Figure 3.5. SDS-PAGE analysis of supernatant from fermenter run #80 having performed pre purification buffer exchange. 20µl of neat supernatant (NS), 20µl of the 1XBB wash (1BB, and 10 and 20 µl of 10mM and 200mM Imidazole elution fractions. 20µl of supernatant flow through (FT) alongside 1,2 and 5 µg of GNA standards by which protein was quantified. The higher molecular weight bands are unknown but thought to be yeast proteins carried over from purification.

Lyophilised protein powder from run #80 to which pre-purification slow buffer exchange was carried out was visually quantified by SDS-PAGE gel to contain 50% intact fusion protein. This was the basis that 10 µg of resuspended protein powder was loaded it corresponded to 5 µg of recombinant GNA (figure 3.6). However, in powder from run #80 there were also high levels of proteins running at a higher molecular weight, especially between 35-43 kDa (figure 3.5, figure 3.6) in comparison analysis of protein from previous fermenter runs #77 and #78 (figure 3.3, 3.4). The use of slow buffer exchange ultimately increased the percentage recovery of protein from 6.7% to 13.7% (table 3.2).

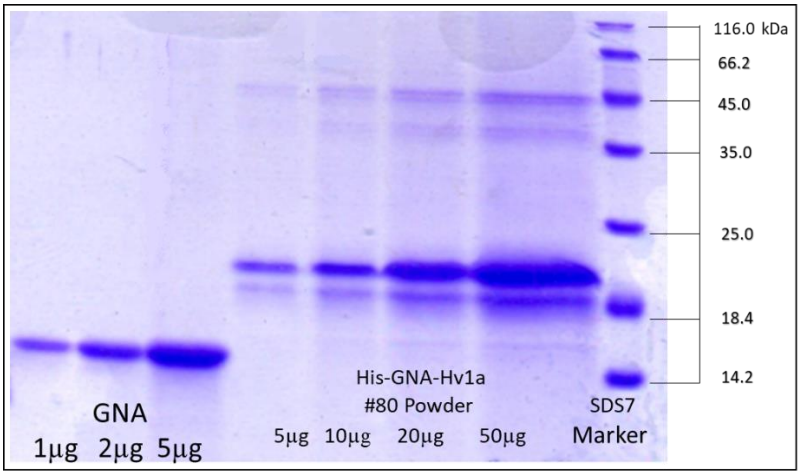

Figure 3.6. SDS-PAGE protein quantification of purified lyophilised His-GNA-Hv1a from fermenter run #80 of His-GNA-Hv1a. Powder was re-suspended at 5mg/ml and quantified to be 50% intact fusion protein against GNA standards of 1, 2 and 5µg. The higher molecular weight bands are unknown but thought to be yeast proteins carried over from purification.

Table 3.2. Table summarising, the amount and percentage recovery of intact fusion protein from His-GNA-Hv1a from fermenter run, #78 where pre-purification buffer exchange wasn't used and #80 where it was.

Fermenter Run	Expression Level mg/L	Usable Supernatant Volume (litres)	Amount Lyophilised Powder (mg)	Total protein recovered (mg)	Percentage recovery of protein from usable supernatant (%)
#78	20	3.5	18.7	4.68	6.7%
#80	50	2.5	34.2	17.1	13.7%

3.3 Discussion

3.3.1 Expression of His-GNA-Hv1a

The expression level of fermenter run #80 of 50 mg/L for His-GNA-Hv1a is good. However, this expression level was still lower than the previous fermentation runs of His-GNA-Hv1a, whose expression level was in excess of 75 mg/l (Morrell, 2016). This lower expression could have been accounted for by the natural variation in the growth of *Pichia pastoris* or the cell density of the starter cultures.

For this work, fermentation of His-GNA-Hv1a in *Pichia pastoris* was run at pH 5. Running the fermenter at an increased pH of 5.5 may have increased expression of stable protein and importantly produced a culture supernatant with a higher pH.

3.3.2. His-GNA-Hv1a Construct

In order to increase the expression of fusion protein from *P.pastoris* and improve the efficiency of protein purification, future work include modifying the existing His-GNA-Hv1a construct without reducing the protein's biological activity.

Future modifications to the α-factor pre pro-secretory signal could be made to increase the efficiency of secretion and increase the amount of protein in the culture supernatant. Future work could introduce multiple His-GNA-Hv1a expression cassettes to *P. pastoris* in order to increase overall expression of the protein. Previously a vector with up to 11 expression cassettes for the fusion protein ModHv1a-GNA-His was generated. Increased yields in *P. pastoris* clones containing up to 9 expression cassettes were seen (Pyati et al., 2014).

The 6X histidine tag has no or little effect on the function of the fusion protein and is essential for purification; an Hv1a-GNA fusion protein without the His tag has a similar level of oral toxicity towards *Mamestra brassicae* larvae (Fitches et al., 2012) as fusion proteins with a His

tag. It has been reported that in some cases the His tag can assist in crystal formation, it is beneficial as it can be used with specific His antibodies in western blot analysis (Carson et al., 2007; Smits et al., 2008).

3.3.3 Pre-purification buffer exchange to raise the culture supernatant pH.

This chapter demonstrates use of slow buffer exchange with 2 XBB, to increase the pH and increase the percentage protein recovery from 6.7 to 13.7%. This coupled with an increased expression level led to a slight increase in the amount of intact fusion protein produced, 17 mg. However, both the percentage yield of fusion protein and total protein produced was still a reduction to that compared to previous fermentation runs of this protein (Morrell, 2016). The lyophilised powder from His-GNA-Hv1a was found to contain 50% intact fusion protein after slow buffer exchange, the same as levels previously reported for this protein (Morrell, 2016).

Pre-purification slow buffer exchange led to the successful increase in supernatant pH and increased the percentage of protein recovered. It also led to a complete reduction of His-GNA-Hv1a present in supernatant flow through after being loaded onto the His-Trap Nickel columns. However, a high level of protein which was not fusion protein, and on weight assumed to be yeast protein was also seen by SDS-PAGE in lyophilised powder. Furthermore, a significant amount of His-GNA-Hv1a protein was eluted in 10 mM imidazole wash, designed only to remove non-specific weakly bound protein. Whilst the Ni^{2+} columns do provide good efficiency to proteins containing histidine residues, the use of a Cobalt resin columns offers higher specificity towards 6-His tagged proteins, with less off-target binding (Bornhorst and Falke, 2000). Cobalt resin columns also release proteins with lower concentrations of imidazole (Bornhorst and Falke, 2000). The introduction of multiple washes with lower concentrations of imidazole to remove non-specifically bound proteins may have been beneficial and led to a cleaner elution fraction. The addition of up to four more histidine residues could increase the affinity of the fusion protein to the Nickel columns; similarly, the use of a dispersed multiple histidine tag can improve stability and increase the affinity to the Nickel columns and aid purification of protein (Bornhorst and Falke, 2000).

At pH 4.75, histidine becomes protonated and is therefore competed off the nickel ion. However, the accessibility of the histidine tag to the column under low pH conditions in the His-GNA-Hv1a could have also been reduced. Raising the pH by slow buffer exchange or using a His tag on the C terminus of the protein may make the histidine residues more accessible and allowed for better column binding.

The use of slow buffer exchange is time consuming and should not be used unless absolutely necessary. However, in this case, due to previous poor recovery as a result of a reduced pH, this method was deemed acceptable. Alternative methods, of using a urea gradient makes use of urea, as a chaotrophic agent at which at high concentrations can bind the histidine residues in

the construct to the divalent metal ion in the column (Ni^{2+}). Refolding by exchange to non-denaturing buffer conditions before elution are then performed. This method has been attempted in past work in the laboratory. However, it is extremely time consuming and has failed due to failure of proteins to refold successfully, particularly those that contain multiple disulphide bonds (Hv1a).

3.4 Summary

This chapter demonstrates the production of the recombinant fusion protein His-GNA-Hv1a by *Pichia pastoris*. It highlights that the design of the protein construct is critical for the ease of purification. It also shows that the use of pre-purification slow buffer exchange can be used when necessary to increase the pH of His-GNA-Hv1a culture supernatant without leading to visible precipitation and that this approach led to improved binding of the protein to the nickel resin thereby enabling an increase in the amount of intact fusion protein recovered. It also suggests that the binding to the His-Trap Nickel columns is sub-optimal in conditions with low pHs.

Chapter 4. Comparison of recombinant fusion proteins produced in *Pichia pastoris*: Expression analysis and characterisation of His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, and Mod-GNA-His.

4.1 Introduction

Hv1a/GNA fusion proteins expressed in *P. pastoris* are toxic to species of Lepidopteran and Hemipteran insect orders (Fitches et al., 2004, 2012, Nakasu et al., 2014). However, fusion proteins containing a single Hv1a toxin peptide and GNA show at best a 50% ratio of intact to cleaved protein (Fitches et al., 2012).

At the C terminal end of the Hv1a toxin peptide, the -VRKE- amino acid contains two dibasic amino acids, lysine and arginine. These two dibasic amino acids make the -VRKE- sequence - very similar to another sequence, -EKRE-. The –EKRE- sequence is targeted for cleavage by the KEX2 gene product also present in *P. pastoris*. In order to reduce cleavage by the KEX2 gene product and increase the proportion of intact fusion protein produced by *P. pastoris,* a protein variant was produced. Producing this variant involved introducing a point mutation in Hv1a, at the 34th amino acid, lysine (K) to produce a glutamine (Q), producing the sequence -VRQE-. This variant is referred to as the K-Q change and fusion proteins with the K-Q modification, for example ModHv1a contain up to 75% of intact fusion protein (Pyati et al., 2014).

This chapter will attempt to characterize the expression of four Hv1a/GNA fusion protein variants: His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, and Mod-GNA-His produced in *P. pastoris*. It will also allow future comparisons to be made against protein expressed in *P. pastoris* with GNA based fusion proteins transiently expressed in *Nicotina benthamiana*.

4.2.1 Protein Constructs

The fusion protein constructs were designed and generated by P. Pyati. His-GNA-Hv1a and His-GNA-ModHv1a, are orientated such that Hv1a is present at the C terminus of GNA. The constructs Hv1a-GNA-His and ModHv1a-GNA-His have Hv1a variants at the N terminus of GNA. The Hv1a toxic peptide is linked to the GNA peptide by a tri-alanine linker region in all variants (Figure 4.1). The modified 6 amino acid His tag has little effect on function as shown by comparable activity in fusion protein activity studies on *M. brassicae* by Fitches et al., 2012.

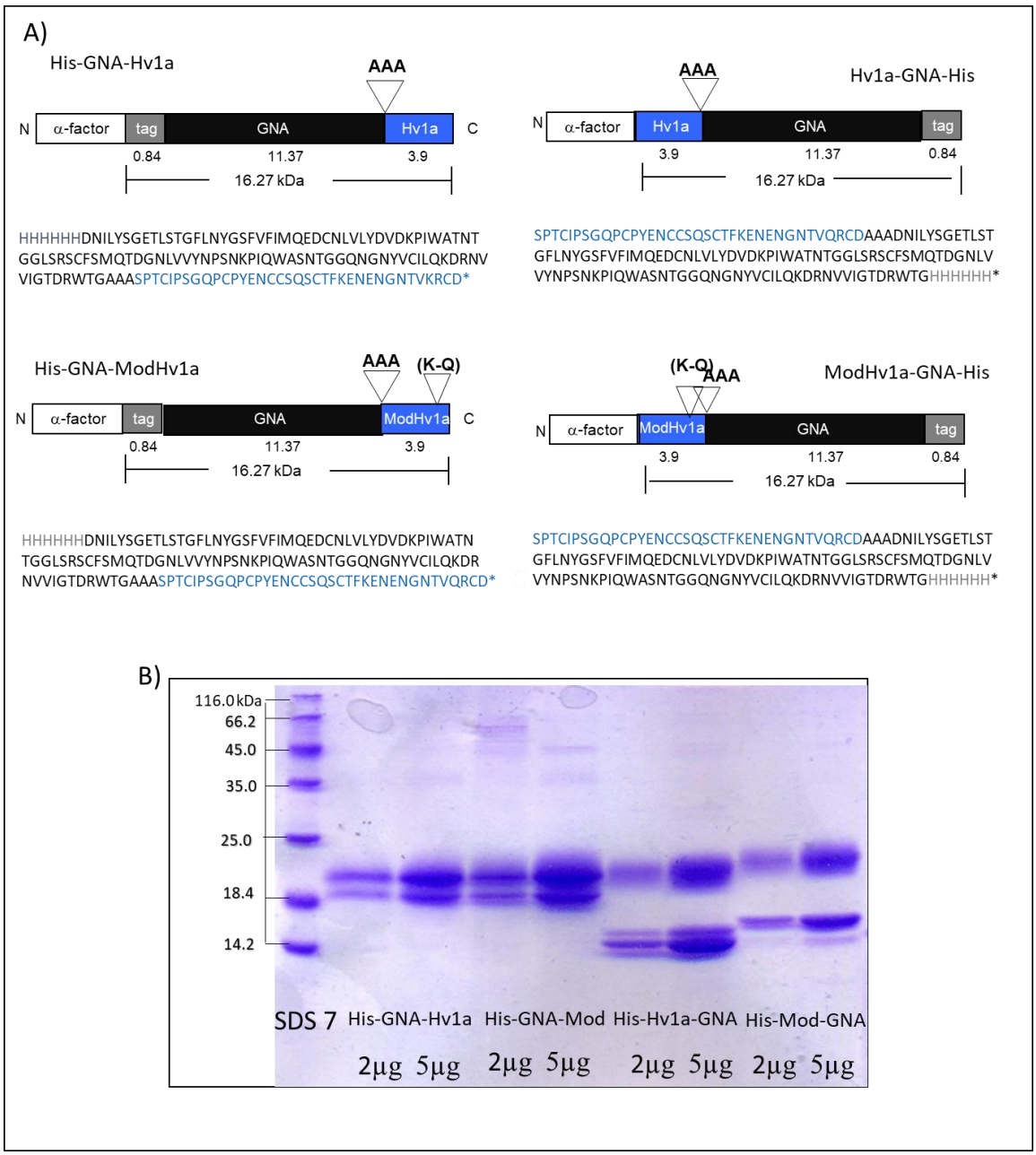

Figure 4.1. A) *Diagrammatic representation of His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His with their theoretical molecular weights as calculated by ExPasy. The alpha factor is highlighted in white, the His-tag is grey, GNA is labelled in black and the Hv1a variant peptide in blue. Below each diagrammatic image is the corresponding protein peptide sequence, with the various colours corresponding to the peptide sequence. B) An SDS-PAGE gel is included showing the band pattern of each protein variant on gel alongside SDS7 a molecular weight marker. Proteins run at a higher molecular weight than expected, thought to be due to the presence of glycosylated proteins.*

The predicted molecular weights for His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His are the same, all 16.27 kDa. However, all the bands for the intact fusion protein run on an SDS-PAGE gel at a higher molecular weight than this, with the hypothesis for this band shift due to the fusion proteins being glycosylated (Figure 4.1B).

4.2.2 Expression analysis

The lyophilised protein powder used in this chapter was produced by Ms B. Morrell (2016) or myself as described previously in this thesis (Chapter 3). Stock protein stocks were re-suspended in 20 mM Sodium Phosphate Buffer pH 7.4 (SPB) when used.

Both His-GNA-Hv1a and His-GNA-ModHv1a run as two clearly distinct, yet tightly spaced bands on a 17.5% SDS-PAGE gel with Coomassie Brilliant Blue (CBB) staining (figure 4.1B). The SDS-PAGE gel band pattern is the same for His-GNA-Hv1a as it is for His-GNA-ModHV1a, with the upper bands runs at 20 kDa and the lower bands run at 18.4 kDa. Furthermore, all bands run at a higher molecular mass than that predicted based upon amino acid sequence.

Hv1a-GNA-His, and ModHv1a-GNA-His, both run as three bands (Figure 4.1B). All bands of ModHv1a-GNA-His run at a slightly higher molecular weight, than the corresponding bands in the non-modified version of the protein. The bands of Hv1a-GNA-His run at 20, 16 and 14.2 kDa respectively and the bands of ModHv1a-GNA-His run at 21, 17 and 15 kDa respectively. The lowest band in terms of molecular weight for ModHv1a-GNA-His, is only visualised on an SDS-PAGE gel when 5µg of intact fusion protein is loaded with the amount of protein in this band is greatly reduced compared to the lowest band in Hv1a-GNA-His (figure 4.1B).

Re-suspended protein has previously been analysed by Western Blot with specific GNA and His antibodies (Figure 4.2). All protein bands were comparable in molecular weight to those seen on an SDS-PAGE gel with CBB staining and were reactive all both GNA and His antibodies, when 200ng of intact fusion protein had been loaded.

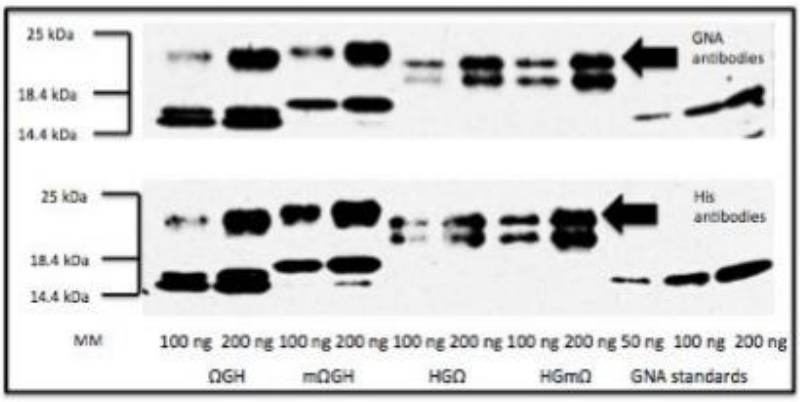

Figure 4.2. Western blot analysis of His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His fusion protein using GNA and His specific antibodies. Recombinant GNA is also loaded at 50, 100 and 200ng of intact protein. (Figure taken from Morrell, 2016).

4.2.3 Glycosylation analysis

As all fusion proteins ran at a higher molecular weight than predicted, periodic acid Schiff (PAS) staining was carried out on His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His to determine whether the recombinant fusion proteins were glycosylated. A further fusion protein (FP6) was used as a positive control. FP6 containing PI1a, a peptide derived spider venom of the *Pireneitega luctuosus* fused to GNA, contains a putative N-glycosylation site (N-X-S/T) at the 35th Asn (Yang et al., 2014). Recombinantly produced GNA was used as a negative control. A magenta colour is seen for all GNA/Hv1a fusion protein variants and FP6 confirming that all the postulated intact proteins were glycosylated.

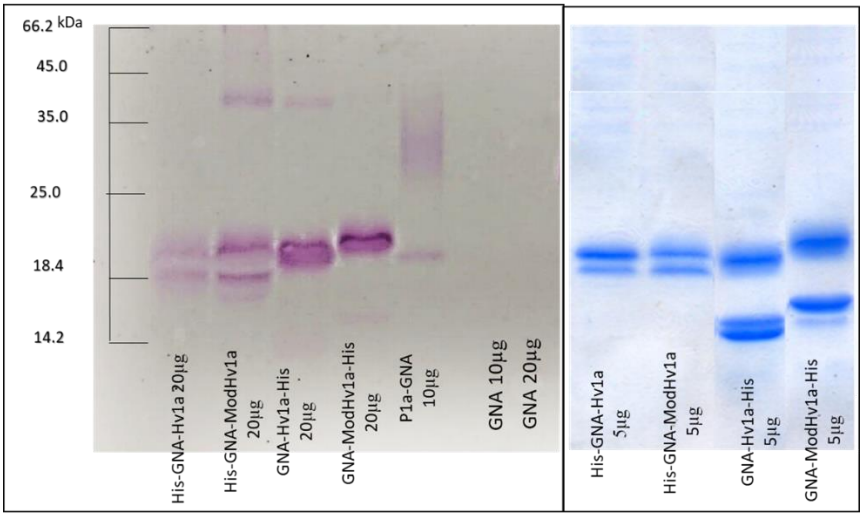

Figure 4.3. *Periodic Acid Schiff staining to visualise glycosylated proteins in His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His protein samples. Intact fusion protein of 20µg was loaded onto an SDS-PAGE gel before being blotted onto a Nitrocellulose membrane. PI1a-GNA (FP6) is loaded at 20 µg as a positive control, with* recombinant *GNA at 20µg also loaded.*

In order to further characterize the nature of this glycosylation, enzymatic deglycosylation reactions on the proteins His-GNA-Hv1a and Hv1a-GNA-His were carried out for 72 hours and proteins run on a 17.5% SDS-PAGE gels and stained with CBB (figure 4.4). Previous deglycosylation reactions were attempted by Morrel., 2016 on His-GNA-ModHv1a and ModHv1a-GNA-His meaning only His-GNA-Hv1a and Hv1a-GNA-His were tested. The reason behind testing these two proteins was that it would allow comparison of two fusion proteins where the toxin is present either side of GNA. N-linked glycosylation is the attachment of a glycan to an asparagine (asn) residue in a protein and almost always attaches to an Asn-X-Ser/Thr sequence where X can be any amino acid other than proline. N-linked deglycosylation reactions were carried out using by N-glycosidase F, which cleaves all asparagine-linked resides, hybrid or high mannose oligosaccharides unless α1, 3-core is fucosylated.

There is no single enzyme that removes O-typed glycans, so a series of exoglycosidases and amindases were used (Endo-α-N-acetygalactosaminidase, α2-3, 6, 8- Neuraminidase, β1,4-Galctosidase, and β-N-Acetlyglucosaminidase).

There was no visual shift in molecular weight seen in the SDS bands the bands of His-GNA-Hv1a and Hv1a-GNA-His post N- or O-linked deglycosylation reactions. However, there was a change in the SDS band pattern for PI1a-GNA which acted as a positive control (figure 4.4).

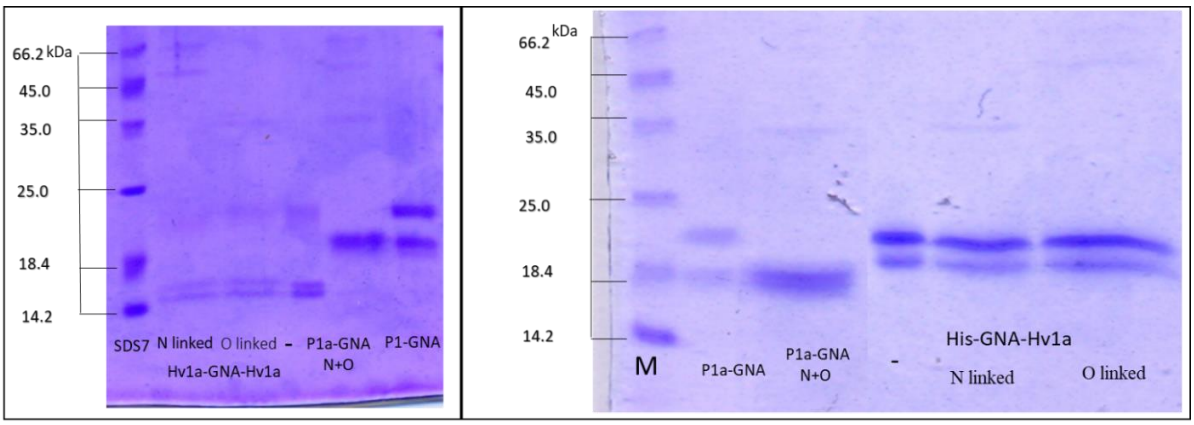

Figure 4.4. *Deglycosylation reactions on His-GNA-Hv1a and Hv1a-GNA-His using N-glycosidase F and a series of O-linked were left to incubate for 72 hours. P1a-GNA (FP6), was used as a positive control and was de-glycosylated.*

4.2.4 N-terminal Sequencing

In an attempt to characterize the sequence of the protein of Hv1a-GNA-His for the bands present on a SDS-PAGE gel, N terminal sequencing had been carried out. To do this, Hv1a-GNA-His had been separated by SDS-PAGE and electro-blotted onto a PVDF membrane (0.15A, maximum voltage for one hour). After the membrane was stained, N terminal sequencing was conducted at Cambridge Biosciences.

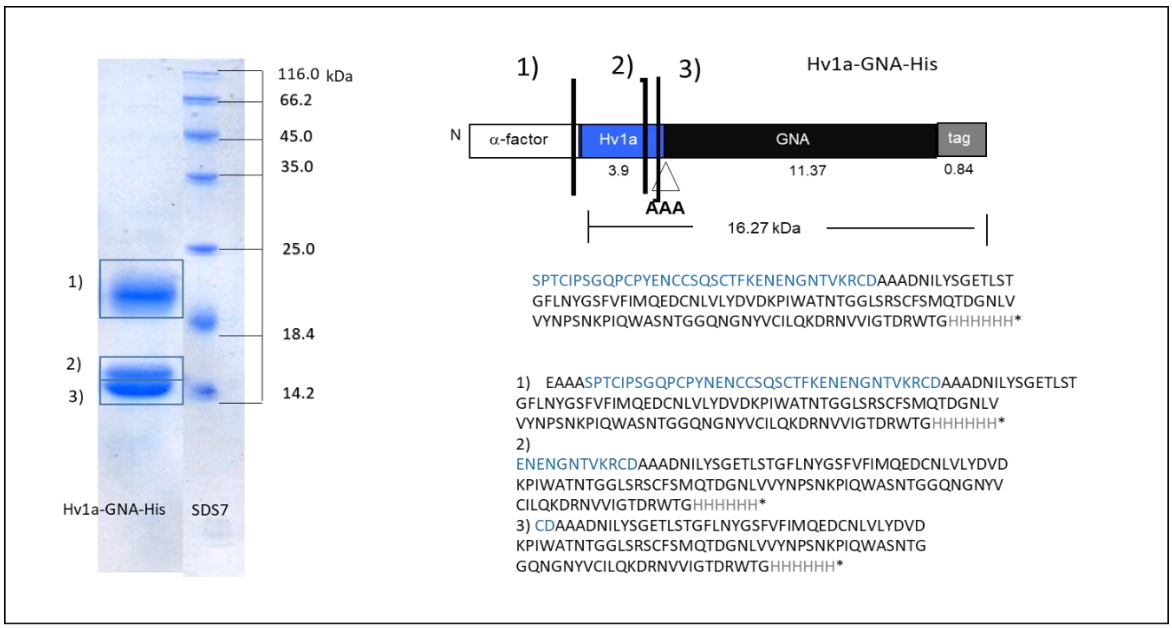

Figure 4.5. N-terminal sequencing was carried out on the three bands labelled 1), 2) and 3) isolated from an SDS-PAGE gel of the Hv1a-GNA-His fusion protein. A diagrammatic representation of the construct shows the sites where N-terminal peptide sequences were matched. Band 1) runs as the highest molecular weight and N-terminal peptide sequences show the peptide sequence starts at the amino acid EAAA, in the alpha factor of the fusion protein construct. Band 2) the middle band on the SDS-PAGE gel shows the peptide starts ENEN at the C terminus of Hv1a, and the peptide isolated from Band 3) begins with CDAA at C terminus of Hv1a two amino acids before the triple alanine linker region .

N-terminal sequencing data suggests that cleavage of Hv1a-GNA-His occurs in two places (Figure 4.5), with both cleavage sites at the C terminus of the Hv1a toxin at amino acid residues 25, (K and E) and amino acid residue number 35, between K and R), the position of the –VKRE- site targeted by the KEX2 cleavage site and K-Q modification in ModHv1a-GNA-His.

4.2.5 Q-Star analysis

Q-Star analysis had previously been carried out by Dr Pyati, and Dr Adrian Brown. Protein bands that had been excised from an SDS-PAGE gel were subject to trypsin digests before being dried by vacuum centrifugation and then dissolved in 10 µl of 0.1% formic acid. They were then analysed using an Applied Biosystems QStar PULSARi™ time of flight mass spectrometer which was coupled to an LC Packings UltiMate™ nano HPLC workstation and results compared against a sequence database compiled by Dr Adrian Brown, Durham University Proteomics.

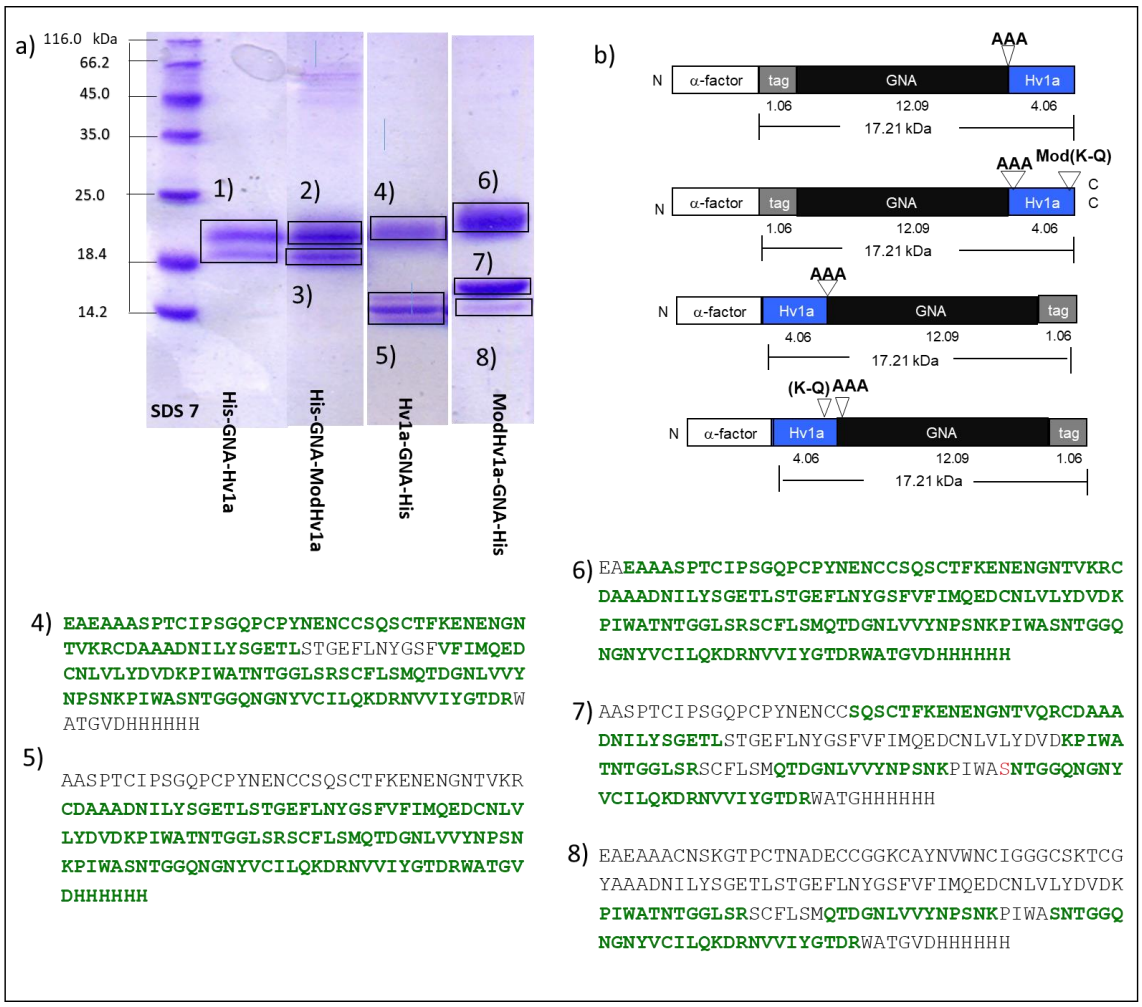

Figure 4.6. a) SDS-PAGE gel depicting the band pattern for each fusion protein and numbered bands representing those that were sent for Q Star analysis. b) Diagrammatic representations of protein constructs which were run on the gel. c) Matched sequences as found by Qstar with the corresponding band number highlighted in green. Bands 4) and 5) come from Hv1a-GNA-His and bands 6), 7) and 8) are from the ModHv1a-GNA-His protein. Where peptide matches were not found this sequence is in grey.

Both protein bands from His-GNA-Hv1a were sent for Q-Star analysis together, as separating them on an SDS- PAGE gel was difficult (Band 1, Figure 4.6). Only GNA peptide matches were found, not matching any predictions (data not shown),and no conclusions can be drawn from the results.

His-GNA-ModHv1a also runs as two glycosylated protein bands on an SDS-PAGE gel, with top band predicted to contain intact fusion protein. Q-Star analysis found peptide matches for both GNA and Hv1a peptides in the higher molecular weight band (Figure 4.6). Based on molecular weight of the lower band of His-GNA-ModHv1a and the fact it is reactive to GNA and His antibodies, it is predicted to be cleaved protein. The cleavage site is expected to be at the C terminus of the toxin. However, this was not confirmed for Q-Star analysis, with Q Star peptide matches found against GNA but not Hv1a (data not shown).

At the time Q Star analysis was undertaken, Hv1a-GNA-His, the top and the two lowermost combined protein bands were sent for Q-Star sequencing as separating the lower bands was tricky. The top protein band of Hv1a-GNA-His is predicted to contain intact fusion protein; (band 4, Figure 4.6) Hv1a and GNA peptides were matched during Q-Star analysis, however no histidine residues were matched at the C terminal end; yet this protein is immunoreactivie by western blot to His reactive antibodies. In two the combined lower two bands (Band 5, figure 4.6), histidine residues were matched during Q-Star analysis, with cleavage seen at VKR-CDAAA. N-terminal sequencing had been subsequently carried out using all three bands also showed cleavage VKR-CDAAA (Figure 4.5).

The top protein band of ModHv1a-GNA-His, is predicted to be intact fusion protein. Q-Star analysis of the upper band has peptide matched the whole sequence of the intact fusion protein. However, it also contains a peptide match for the amino acid sequence –EAAA-, which is present at the end of the alpha factor and suggests that the alpha factor is not cleaved completely, this is in line with N-terminal sequencing data. The 1st cleavage product runs at 18kDa and has spectrum matches with the majority of GNA and some matches in Hv1a, however nothing conclusive about the N or C terminus can be made. The second cleavage product running 14.5kDa was not predicted to contain intact fusion protein, and Q star data shows only matches with GNA.

4.2.6 Solubility of lyophilised powder containing the four Hv1a-GNA fusion protein variants.

The solubility of fusion protein variants was assessed when suspended in SPB. A small pellet is observed when lyophilised Hv1a-GNA-His powder is re-suspended at 5mg/ml and was quantified to contain only 10% fusion protein. All other proteins dissolved well at 5mg/ml with no pellet being seen after centrifugation at 13,000 rpm for 1 minute.

4.2.7 Quantification of the lyophilised protein powder used.

Lyophilised protein was re-suspended in SPB and quantified by visual analysis using an SDS-PAGE gel which had been stained for total proteins with CBB. The visual quantification of intact fusion protein was measured against the intensity of CBB of recombinant GNA standards, of 1, 2 and 5µg of protein (Figure 4.7). Correct quantification was important for all subsequent work described in this chapter and indeed throughout the rest of this thesis.

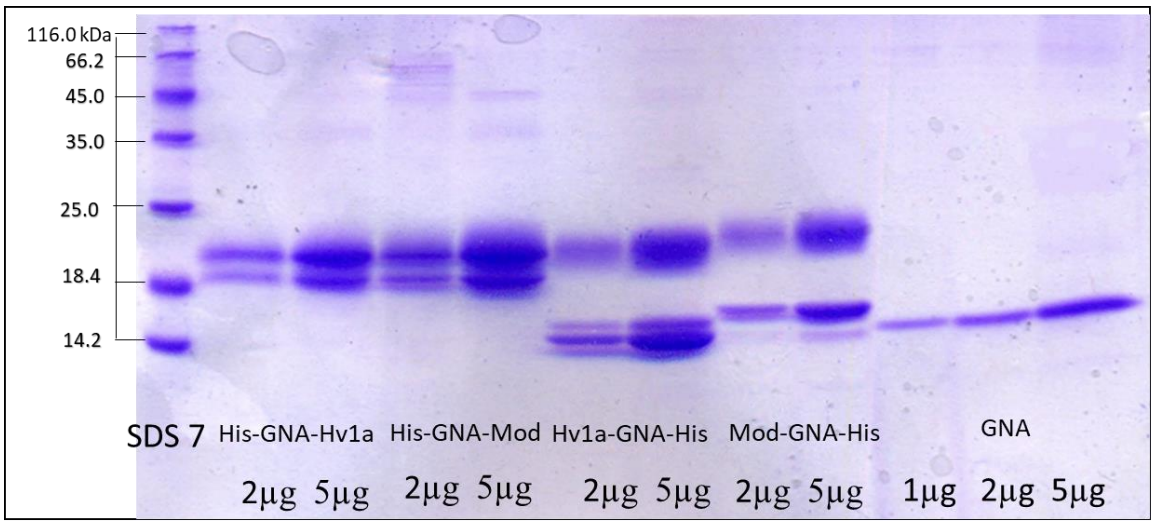

Figure 4.7. Lyophilised purified proteins produced by Morrell; powders were re-suspended in SPB and quantified by visual comparison of band intensity staining against known recombinant GNA standards of 1, 2 and 5 µg. 2µg and 5µg of intact fusion protein were loaded.

Lyophilised powder of His-GNA-Hv1a from fermenter run #49 was quantified as 50% (w/w) of intact fusion protein. SDS-PAGE (figure 4.7) analysis suggests that cleaved fusion protein makes up the majority of the rest of the lyophilised powder with carbohydrates also contributing to this.

His-GNA-ModHv1a from fermenter run #56 was quantified at 30% (w/w) intact fusion protein with the remaining 70% of powder made up of cleaved protein, higher molecular weight proteins and carbohydrates (figure 4.3). These higher molecular weight proteins can be viewed faintly on SDS-PAGE gel with CBB staining (figure 4.7) and give positive results to PAS staining (figure 4.3), confirming they are glycosylated. It is likely that these are therefore endogenous yeast proteins that may contain histidine residues.

Hv1a-GNA-His powder was only quantified as 10% (w/w) protein, with high levels of carbohydrates presumably making up the rest of the powder.

The lyophilised powder containing the fusion protein, ModHv1a-GNA-His was quantified to contain 40% (w/w) intact protein, with the majority of the remaining powder containing cleaved protein product.

4.3 Discussion

4.3.1 Characterisation of protein expression, by compiling analysis by SDS-PAGE electrophoresis, Western blot, Q-Star and N Terminal Sequencing data.

Analysis of SDS-Page gels and Western blots shows that all four fusion protein variants have been subject to cleavage to some extent, with intact fusion protein running at approximately 22

kDa, higher than predicted molecular weight of 16.2 kDa. Proteins all displayed expression profiles as previously reported by Morrell, (2016).

Analysis of SDS-PAGE gel bands by Q-Star, N terminal sequencing and visual analysis is difficult, as each band likely to contain numerous different protein fragments. All protein bands from the four fusion protein variants were reactive to both His and GNA specific antibodies. If GNA was cleaved at any point then a positive result to a GNA specific Western blot is unlikely to have been seen, however it does not rule out the possibility that the antibody was raised against a particular region of the GNA protein that was present in a cleaved product. Together with mass spectrometry analysis, it suggested that cleavage must occur within the Hv1a toxin.

N-terminal sequencing analysis on His-GNA-Hv1a and His-GNA-ModHv1a would provide additional information as Western Blot analysis shows that the His tag and GNA are present in all bands, therefore N-terminal sequencing would only confirm this. However, the His-GNA-Hv1a and His-GNA-ModHv1a samples used for Q Star have ionised poorly and could be repeated with both bands to be sent for analysis.

His-GNA-Hv1a and His-GNA-ModHv1a run as two bands on an SDS-gel. It is hypothesised that their cleavage site is similar to that in Hv1a-GNA-His, at the C terminus of the Hv1a toxin at amino acid residues 25, (K and E) as shown by Hv1a-GNA-His N-terminal sequencing.

N terminal sequencing of Hv1a-GNA-His and Q Star data of ModHv1a-GNA-His suggests that there is incomplete processing cleavage of pre pro protein sequence, at –EAEAAA- with spectrum matches for –EAAA-. N terminal sequencing on ModHv1a-GNA-His could confirm this and may also elucidate why ModHv1a-GNA-His runs at a higher molecular weight, although there are other factors that could explain why the fusion proteins do not run at their predicted molecular masses (Chapter 4.3.2)

4.3.2 Glycosylation analysis

Glycosylation is recognized as one of the most common forms of post translational modifications that proteins undergo (Khoury et al., 2011). The presence of N- linked sugars in yeast is common and often seen in recombinant protein production (Bretthauer,and Castellino, 1999) Whilst O-linked glycosylation has been estimated to account for approximately 12% of glycosylation's in all yeast proteins (Goettig, 2016). The evidence for the presence of O-linked glycosylation in *Pichia pastoris* is limited as compared to the more common N- linked glycosylations; yet have still been shown to occur and have important functions in preventing general and specific proteolysis (Bretthauer,and Castellino, 1990).

Previously it had been shown that His-GNA-Hv1a and His-GNA-ModHv1a were not N-linked glycosylated and this is not surprising given they don't have the N-X-T sequence that signals N-linked glycosylation (Morrell, 2016). However, one hypothesis that the difference between the

distinct yet tight spacing bands of 20kDa and 18.4kDa of both His-GNA-Hv1a and His-GNA-ModHv1a protein samples could be due to intact protein being differentially glycosylated. PAS staining shows the intensity of staining of both bands are similar, suggesting similar levels of glycosylation. From the results it cannot be concluded that the proteins are differentially glycosylated, with deglycosylation reactions using the same N and O-linked glycosidases showing no change in molecular weight of either band in His-GNA-Hv1a.

Glycosylated sugars attached to all the fusion protein variants, as confirmed by Schiff-PAS staining will add mass to the proteins. Therefore, this could account at least partially for the proteins running on SDS-PAGE gel at molecular weights higher than predicted by amino acid sequence.

Unlike N-Glycosidase F, no one commercially available enzyme is as efficient for removing O-linked glycosylations for which there is no consensus sequence. Evidence shows that the O-linked glycosylation sites in *P. pastoris* are generally less than 5 residues in length and generally shorter than those produced *in S. cerevisiae* (Bretthauer and Castellino, 1999). In addition to the enzymes supplied by the Calbiochem, glycoprotein deglycosylation kit used here, further enzymes are required for complete O-linked deglycosylation. Furthermore, if fucose and mannose are O-linked indirectly, there is currently no way that these sugars can be removed enzymatically. To remove all O-linked glycans completely, chemical methods must be followed, one of the most commonly employed techniques is hydrazinolysis and alkali/reducing conditions (β-elimination). Originally found to be used for N linked glycosylations, use of anhydrous hydrazine for release of O- linked glycans is also now possible.

Not all of the protein bands of Hv1a-GNA-His and ModHv1a-GNA-His show positive results for glycosylation after PAS staining. However, there are lower intensity pink bands visible, but from this analysis it is impossible to tell if they relate to the second or third cleavage fragment suggesting a reduction in sugar content in this cleaved band. PAS staining only requires 0.2µg of protein-associated carbohydrate for a positive result (Doerner and White, 1990), the lack positive results either the 2nd or 3rd cleavage products of Hv1a-GNA-His and ModHv1a-GNA-His at 20 µg suggest they are not glycosylated.

Q-Star analysis and N-terminal sequencing alongside Western Blot analysis have shown that the cleaved protein bands of Hv1a-GNA-His and ModHv1a-GNA-His contain mainly GNA fragments. Recombinant GNA shows no reactivity to PAS staining, considering GNA is a recombinant protein is may be surprising. However, GNA contains no asparagine residues so N-linked glycosylation would not be expected. The cleaved protein bands of Hv1a-GNA-His and ModHv1a-GNA-His show a faint positive result with Schiff- PAS staining leaving a faint pink band and is evidence of reduced sugar content.

4.3.3 Quantification of intact His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA from protein powders

The work done in this chapter relies heavily on accurate quantification of protein against GNA recombinant standards. Previous work in the laboratory has found that BCA Protein assay kit and Bradford assays do not provide protein estimates that correlate well with visual estimates based on SDS-PAGE analysis. This is generally because BCA and Bradford assay kits calculate the total protein content assayed, but as demonstrated in this thesis these recombinant fusion proteins contain more than one peptide. Furthermore, post translational modifications in particular the presence of glycosylated sugars affects the ability of BCA and Bradford assay kits to accurately estimate the total protein content (Brady and Macnaughtan, 2015). The development of imaging software where band size and pixel intensity are used to compare bands to in range GNA standards of a known quantification would have been beneficial as the best current software available (ImageJ) only takes into account band size.

4.4 Summary

The expression pattern by SDS-PAGE and characterisation of cleavage products has been partially characterized for His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His fusion protein variants. Future N-terminal sequencing would not be suitable for characterizing the His-GNA-Hv1a and His-GNA-ModHv1a nor would future Q-Star analysis using trypsin enzymes for digest.

Chapter 5. Biological activity of His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His recombinant proteins produced by *Pichia pastoris*

5.1 Introduction

The phloem feeder, *Acyrthosiphon pisum*, of the insect order Hemiptera causes major damage to crop plants. The intensive use of chemically produced pesticides has positively selected aphid genotypes resistant to organophosphates which inhibit acetylcholinesterase's (Devonshire et al., 1998). It has also led to resistance to pyrethroids which target sodium channels via mutations in ion channel binding sites and as a result a novel class of insecticides is required (Devonshire et al., 1998).

The recombinant Hv1a/GNA fusion proteins produced in *Pichia pastori*s have been demonstrated to be active against lepidopteran and dipteran insects via injection directly into the haemolymph (Yang et al., 2015). Although fusion proteins are toxic by injection, it is not viable to inject insects individually in a real-world setting. Fusion proteins must be toxic orally or by contact to be viable as an insect control strategy. Aphid bioassays with Hv1a/GNA fusion proteins have shown that these fusion proteins are toxic by ingestion. (Fitches et al., 2012, Morrell, 2016).

In order to assess if the biological activity of the GNA was retained in these protein samples, a haemagglutination assay was conducted with fresh rabbit erythrocytes. This assay allowed the assessment as to whether the position of GNA relative to the toxin in the fusion protein affected the agglutination activity of GNA. The toxicity of the four fusion proteins, His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His was also tested with oral bioassays against *A. pisum*. Subsequently the stability of the four fusion proteins in the aphid diet was also assessed *in vitro*.

5.2 Results

5.2.1 *In vitro* haemagglutination assay of His-GNA

An *in vitro* haemagglutination of rabbit erythrocytes is a common test of the biological activity of lectins and was used here to assess if the GNA contained in the four fusion protein variants was functional (figure 5.1). If the lectin has agglutination activity the erythrocytes in the assay cross-link to each other and stay suspended in the solution leaving the solution in the well a pale red. If the sample does not possess agglutination activity or are rendered inactive, it causes the erythrocytes in the assay to fall out of suspension and form a dark red pellet which can easily be visualised at the bottom of a 96 well microtiter plate (negative control, Figure 5.1).

The amount of GNA present in each fusion protein variant was calculated using visual analysis of SDS-PAGE blots with the GNA present in cleavage products also considered in the calculations. Analysis of the haemagglutination assay reaction revealed that commercially

available Sigma GNA, recombinant GNA and the four fusion protein variants: His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His were all able to agglutinate rabbit erythrocytes, but to varying degrees as visualised on a microtiter plate (figure 5.1).

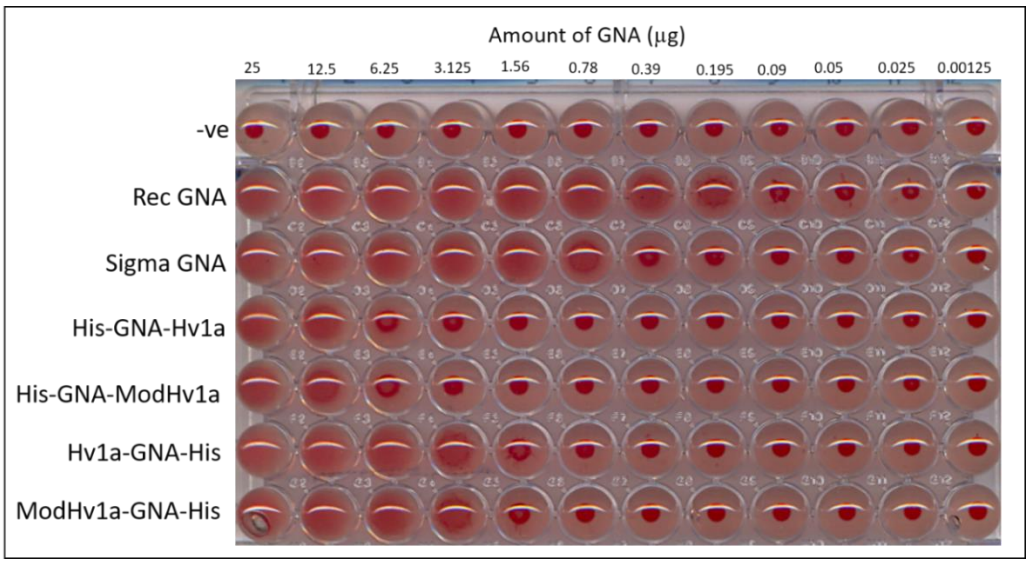

Figure 5.1. Ability of recombinant proteins to agglutinate rabbit erythrocytes. In vitro haemagglutination assay of four fusion protein variants His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His. The negative control consisted of 1X Phosphate Buffer Saline (PBS). 50 µl of 0.1 mg/ml solutions were serially diluted with 5 0µl of 2% (v/v) erythrocyte suspension added to each well. Sigma GNA and Recombinant GNA were both used as a positive control.

All the fusion protein variants tested, agglutinated rabbit erythrocytes to a lower level than the recombinant or GNA alone (figure 5.1). The commercially produced GNA, purified from *Galanthus nivalis* by Sigma was found to agglutinate down to a concentration of 0.39 µg/ml, this was higher than the recombinant GNA samples which agglutinated to 0.09 µg/ml. Hv1a-GNA-His and Mod-GNA-His showed partial agglutination, at a GNA concentration of 3.125 µg/ml. This was superior compared to protein samples of His-GNA-Hv1a and His-GNA-Mod which only showed partial agglutination to a concentration of GNA at 12.5 µg/ml (figure 5.1).

5.2.2 Oral bioassays with *Acyrthosiphon pisum*

Oral bioassays against *A. pisum*, pea aphids, were carried out for the four recombinant GNA/Hv1a fusion protein variants; His-GNAHv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His.

Fusion protein was re-suspended to 5 mg/ml with sodium phosphate buffer (SPB) and checked visually on a SDS-PAGE gel with Coomassie Brilliant Blue (CBB) staining (data not shown). This 5 mg/ml fusion protein solution was then mixed with aphid diet in order to produce differing concentrations, ranging from 0.1 to 1 mg/ml of intact fusion protein. Diet was

regularly checked by SDS-PAGE to ensure the correct amount of fusion protein was contained in each treatment (figure 5.2).

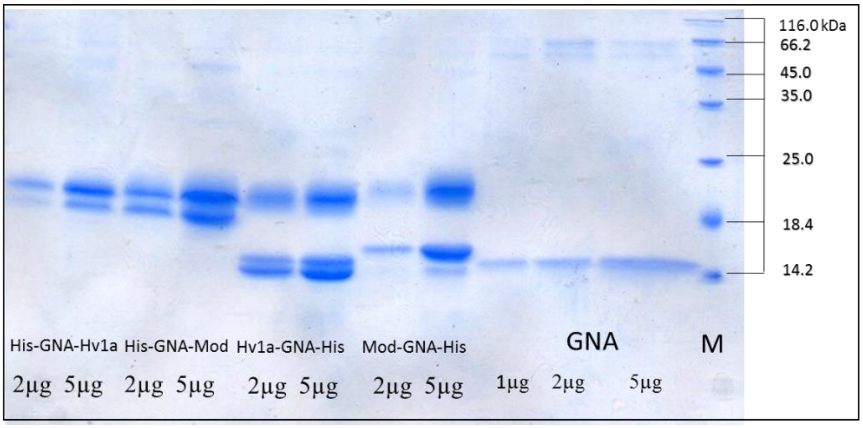

Figure 5.2 SDS-PAGE with CBB staining of aphid diet containing fusion proteins, His-GNAHv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-Hiss loaded at 2 and 5 µl to ensure the correct amount of fusion protein was contained in each treatment. Recombinant GNA was used as a standard.

Aphids were fed on diet containing either 0, 0.1, 0.25, 0.5, 0.75 or 1 mg/ml of intact fusion protein. The number of aphids dead and alive were counted around the same time every day for 10 days, at which point aphid nymphs from controls started to be produced and the assay was terminated. Survival curves were generated for His-GNAHv1a (figure 5.3), His-GNA-ModHv1a (figure 5.4), Hv1a-GNA-His (figure 5.5) and ModHv1a-GNA-His (figure 5.6). LC50 values were then calculated for each fusion protein using day 7 survival data (table 5.1).

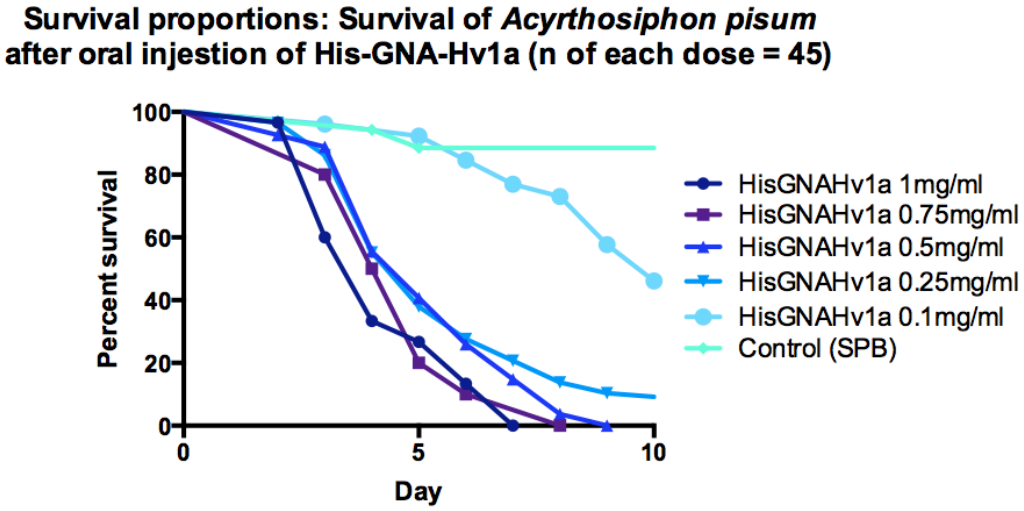

Figure 5.3. Survival curve of Acyrthosiphon pisum after oral ingestion of His-GNA-Hv1a with survival over 10 days recorded. Doses ranged from 0.1 – 1 mg/ml with a sodium phosphate buffer (SPB) control. The number of A. pisum used for each treatment was 45.

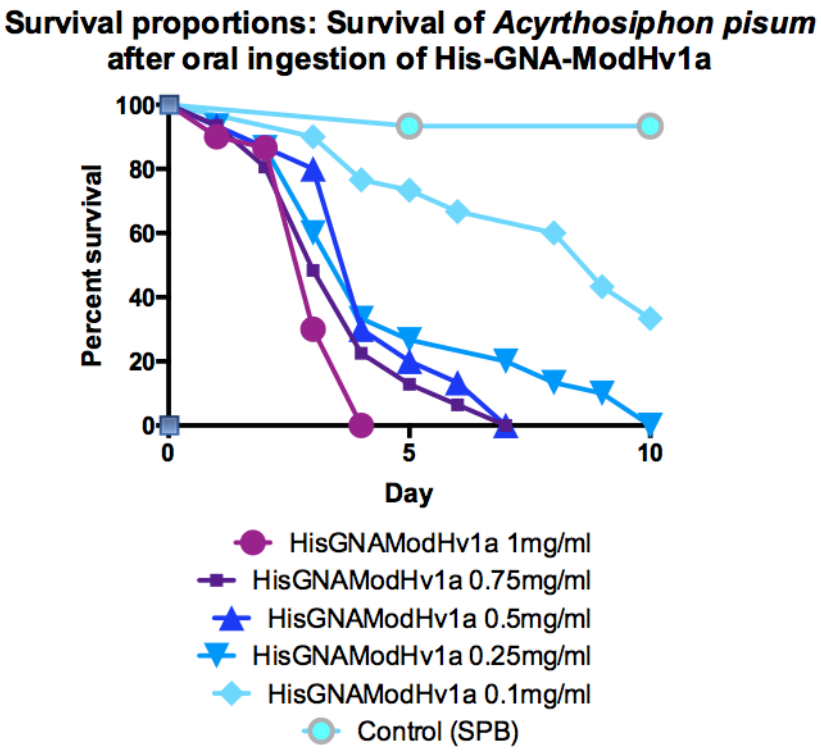

Figure 5.4 Survival curve of Acyrthosiphon pisum after oral ingestion of His-GNA-ModHv1a with survival over 10 days recorded. Doses ranged from 0.1 – 1 mg/ml with a SPB control. The number of A. pisum used for each treatment was 45.

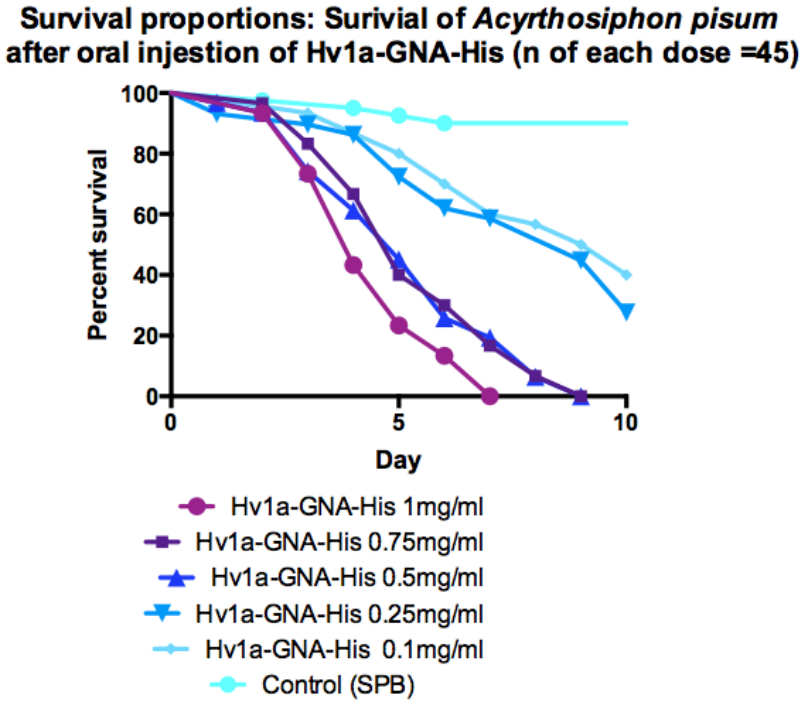

Figure 5.5 Survival curve of Acyrthosiphon pisum after oral ingestion of Hv1a-GNA-His with survival over 10 days recorded. Doses ranged from 0.1 – 1 mg/ml with a SPB control. The number of A. pisum used for each treatment was 45.

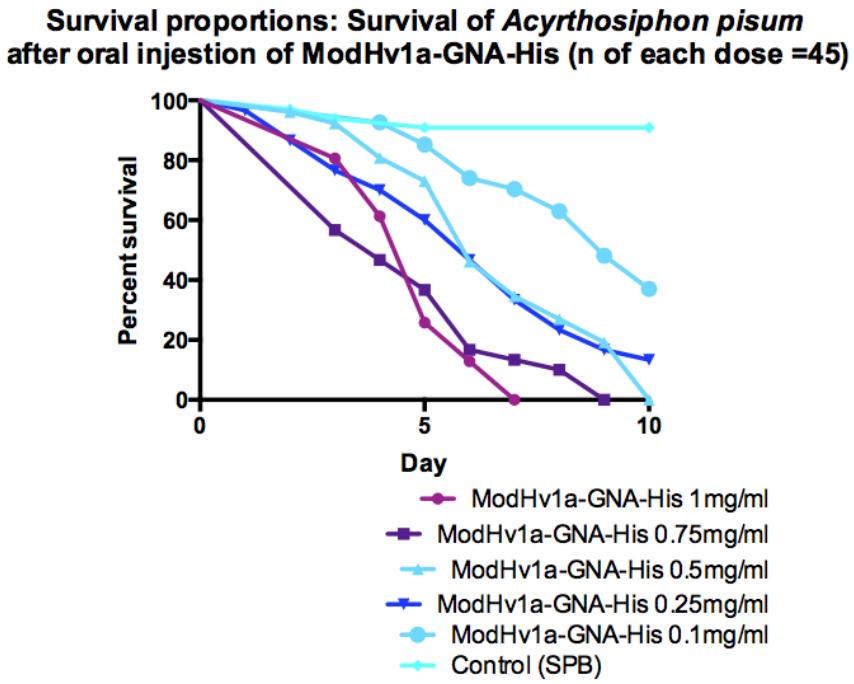

Figure 5.6. Survival curve of Acyrthosiphon pisum after oral ingestion of ModHv1a-GNA-His with survival over 10 days recorded. Doses ranged from 0.1 – 1 mg/ml with a SPB control. The number of A. pisum used for each treatment was 45.

Table 5.1. LC50 values for the four fusion protein variants against *Acyrthosiphon pisum* with values calculated with day 7 survival data.

Fusion Protein	LC50 against *A. pisum (mg/ml)*
His-GNA-Hv1a	0.133
His-GNA-ModHv1a	0.131
Hv1a-GNA-His	0.202
ModHv1a-GNA-His	0.113

All aphids feeding on treatments containing fusion protein that remained alive after 10 days were visually seen to be much reduced in size compared to those feeding on diet and SPB controls although aphids were not weighed or measured.

Results for each fusion protein were dose dependent with higher doses killing more aphids after ten days than lower doses. The LC50 value of 0.202 mg/ml for fusion protein Hv1a-GNA-His was the greatest and this value is relatively similar to LD50 values of the other fusion protein variants, His-GNA-Hv1a, His-GNA-ModHv1a and ModHv1a-GNA-His (table 5.1).

5.2.3 Stability of fusion protein in diet *in vitro.*

A preliminary experiment whereby both diet containing fusion protein, and diet with no active protein was fed to aphids in order to see how much a single aphid consumes within 24 hours.

The amount of liquid diet was then measured by pipette with results showing approximately 0.5 µl of liquid diet was consumed per *A. pisum* with no difference when fusion protein at a concentration of 0.5 mg/ml was fed. This study provided the basis as to how much protein to load to assess the stability of fusion protein *in vitro*.

To investigate the stability of the four fusion protein variants in the diet, a stability assay was carried out. For each sample a total of 15 adult pea aphids, each of similar size were fed on artificial diet at a concentration of 0.5 $mgml^{-1}$. 20 µl of diet was subsequently analysed by western blotting, 24, 48, and 72 hours after being exposed to feeding aphids. After 72 hours there was no degradation of fusion protein seen in the aphid diet or in the control diet which was left for 72 hours without aphids feeding (Figure 5.7).

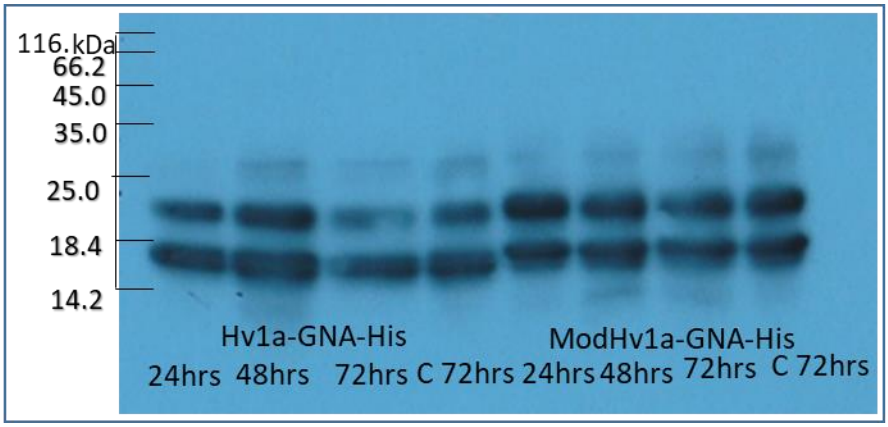

Figure 5.7 Western Blot using His-specific antibodies of Hv1a-GNA-His and ModHv1a-GNA-His diet containing after being subject to feeding on by A. pisum for diet 24, 48 and 72 hours. All samples loaded are 10 µl of liquid diet.

5.3 Discussion

5.3.1 Haemagglutination

Haemagglutination assays demonstrated that the GNA present in re-suspended samples containing His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His is functionally active as they were all able to cross link rabbit erythrocytes. The results corroborate that GNA is still active when fused to either the N or C terminus of the toxin, in line with previous work (Morrell, 2016). The ability of the GNA present in the four proteins discussed in this chapter is similar to that of other fusion proteins previously tested, and comparable to the activity of previous fusion proteins (Fitches et al., 2004, 10).

Although there is reduced agglutination ability of GNA in fusion proteins compared to GNA purified from snowdrop (*Galanthus nivalis*) and recombinantly produced GNA, recombinant GNA retains the ability to bind to the gut epithelium (Fitches et al., 2002). Although the total GNA present as both cleaved and part of the fully intact fusion protein was taken into account in the assay, there is an increased proportion of GNA present as part of the full intact fusion

protein in the His-GNA-Hv1a and His-GNA- samples is compared to ModHv1a Hv1a-GNA-His and ModHv1a-GNA-His. The increased agglutination activity described in this chapter may be due to as yet undescribed level of steric hindrance caused by the toxins when fused to the N terminus of GNA.

5.3.2 Oral Toxicity Bioassay

All four fusion protein variants were toxic towards *A. pisum* by oral ingestion, and is in line with previous work reported by Morrell (2016) although only a single dose of each variant was tested whereas a full dose response was obtained in this study.

All fusion protein variants tested exhibit LC50 values higher than GNA alone which has a degree of insecticidal activity towards pea aphids (day 10 LC50 =0.42 mg/ml, (P.Pyati pers. Comm.). No significant difference ($p>0.05$) is seen between the four fusion protein variants, and no particular difference observed whether the Hv1a toxin was linked to the N or C terminus of GNA or whether the modified Hv1a peptide was used. Future bioassays could also take into account the number of nymphs produced by the aphids which could shed more light on any differences in toxicity between the fusion protein samples.

Carrying out bioassays to calculate the LC50 values of the fusion protein variants against other aphid species including *Mysuz persicae* and *Sitobion avenae* should also be completed, as previous work has shown they are more resistant to the four fusion proteins (Morrell, 2016).

5.3.3 Fusion protein stability

It was hypothesised the slight differences in toxicity of the fusion proteins could be due to the stability of the protein in aphid diet and after ingestion. However, all fusion protein present in the diet after an *in vitro* study was stable for up to 72 hours.

A future *in vitro* study by which the fusion proteins are incubated with homogenised guts and a separate *in vivo* study of the same proteins using gut dissections should be carried out to determine if there are differences in the stability of the four fusion protein variants. Ideally these experiments would have been conducted due to time restrictions were not carried out.

5.4 Summary

In summary this chapter demonstrates that fusion proteins produced recombinantly in *Pichia pastoris* retained their biological activity and toxicity. LC50 values were calculated for His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His against *A. pisum* with no significant difference found between the fusion proteins. The stability of all four fusion proteins was also found to be similar with no degradation of fusion protein in diet seen *in vitro* after 72 hours.

Chapter 6. Production and generation of *Nicotina benthamiana* transiently expressing GNA/Hv1a fusion protein using the Gateway™ cloning system.

6.1 Introduction

Transgenic plants, containing *Bacillus thurigenisis* (Bt) toxins are currently the only commercially available genetically modified (GM) plants used as insecticides. They are efficient against many insects including Lepidoptera, Coleoptera, Hymenoptera and Diptera orders (Lopez et al., 2013). Despite this, Bt toxins are ineffective against certain insect pests including aphids, as these species do not possess the appropriate receptors upon which the toxins act on (Porcar et al., 2009). Furthermore, the development of resistance in insects against plants containing Bt and many exogenously applied insecticides insecticide resistance continues to rise (Brevault et al., 2013) and there is a need to develop a new class of insecticides.

Hemipteran pests, including aphids feed directly from the phloem via a long stylet. A fusion protein expressed *in planta*, ideally in the phloem would be preferable to application by exogenous spraying. Hv1a/GNA fusion proteins display toxicity to Hemipteran when orally digested as displayed in chapter 5 of this thesis; and are also orally toxic towards Lepidopteran orders (Fitches et al., 2012, Yang et al., 2015, Morrell, 2016). This suggests that these fusion proteins are good candidates for expression *in planta*.

An Hv1a-GNA fusion protein, containing a GNA leader sequence has previously been expressed in *Arabidopsis thaliana* under the constitutive promoter, CaM35S (Nakasu et al., 2014b). Western blot analysis of leaf tissue which expressed this fusion protein showed the expression level to be approximately 25.6+-4.1ngmg^{-1} fresh weight leaf tissue (Nakasu et al., 2014b). The protein was found to be functional, and detached leaf tissue was then used in assays which induced up to 40% mortality in the peach potato aphid, *Myzus persicae*. (Nakasu et al., 2014b). This rate of mortality was comparable to that seen in artificial diet bioassays with the Hv1a-GNA protein construct produced in *P. pastoris*.

Work in this chapter acts as a first step to elucidating which is the best fusion construct to take forward for stable expression in *A. thaliana*, through transiently expressing Hv1a/GNA fusion proteins in *Nicotina benthamiana.* This chapter will discuss the cloning programme used for four variant proteins and compare the expression profile of the fusion protein: GNALeader-GNA-ModHv1a produced in *N. benthamiana* to the His-GNA-ModHv1a protein produced in *P. pastoris*.

6.2.1 Fusion protein constructs

Modifications to the four fusion variants described previously in this thesis; His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His and ModHv1a-GNA-His were made for constitutive expression in plants (Figure 6.1). Yet the protein sequence of all four fusion proteins were not

codon optimised for expression in *N. benthamiana* as when compared the sequences were deemed similar enough for efficient expression.

In order to use the GATEWAY™ cloning system modifications to the existing Hv1a/GNA fusion protein constructs (chapter 4) were introduced by PCR (Figure 6.2). Modifications included the removal of the six amino acid histidine tag, as the protein was not purified by metal ion chromatography. In addition a stop codon (with an amino acid sequence, TAG) was introduced to the C terminus of constructs and this allowed for cloning into the destination vector, Pearlygate 201 to occur via a recombinase reaction.

For the GNA peptide to be expressed in planta, the GNA leader sequence must be present, this sequence is processed and cleaved post translation, and in doing so targets the GNA protein to the nuclear or cytoplasmic compartment from the vacuole (Fouquaert, 2007). Therefore, the GNA leader sequence which incorporated a SalI restriction site at the C terminus was synthesised by Shinegene.

The GNA leader sequence was then fused to the GNA-toxin constructs by restriction-ligation reactions, with the restriction enzyme Sal1 and T4 ligase respectively. All PCR products were generated as blunt-ended leaving a four base pair overhang at the N terminus of the GNA leader sequence (CACC), which is required for directional in order to be cloned directionally into the entry (pENTR) vector supplied by Invitrogen.

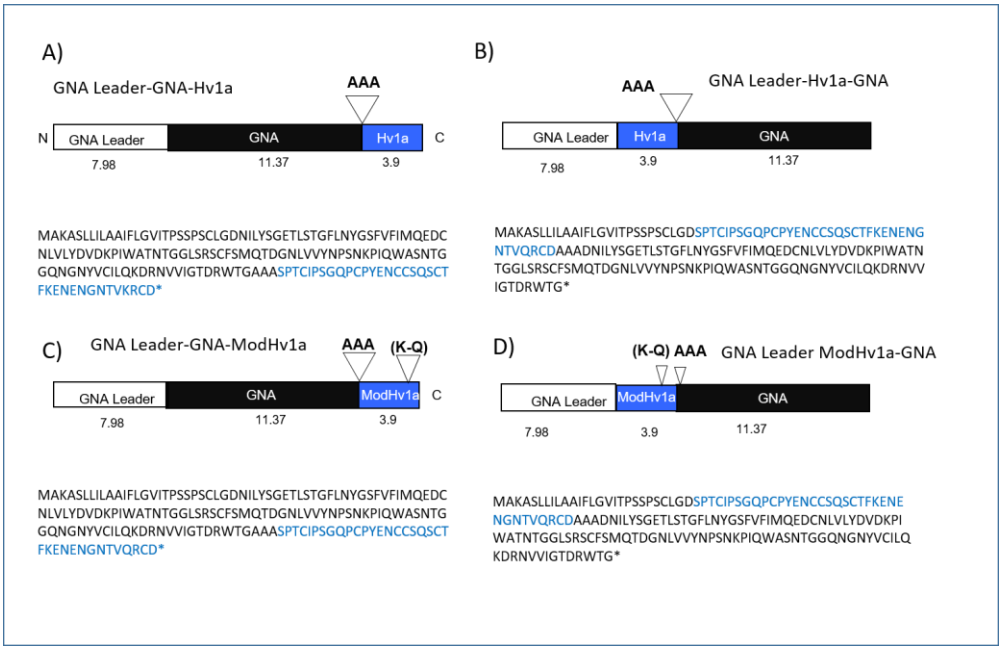

Figure 6.1. *Diagrammatic representation of the constructs introduced into the GATEWAY pEntr vector. A) GNA Leader-GNA-Hv1a, B) GNA Leader-GNA-ModHv1a C) GNA Leader-Hv1a-GNA D) GNA Leader-ModHv1a-GNA. The amino acid sequences are below the corresponding construct with the blue highlighting the toxin component of the fusion protein.*

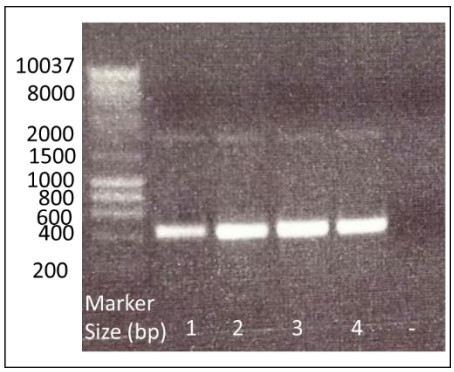

Figure 6.2. *Gel photo after PCR reactions of four fusion protein constructs 1) GNA-Hv1a, 2) GNA-ModHv1a, 3) Hv1a-GNA and 4) ModHv1a-GNA before restriction-ligation reactions.*

Successful ligation reactions were then subject to a PCR amplification reaction before DNA was extracted from the 1% agarose gel, and subject to ethanol precipitation. This DNA was then inserted into pJET cloning vectors using T4 ligase and a pJET cloning kit (Thermo) and plasmids were subsequently electroporated into *Escherichia coli* Top10 cells. The transformed *E. coli* cells were left to grow overnight on LSLB plates containing carbenicillin, positive colonies were confirmed by PCR were stored as glycerol stocks.

6.2.2 GATEWAY™ cloning system

The DNA from the PCR reactions was cleaned and purified by ethanol precipitation before GATEWAY™ D-TOPO reactions were carried out, as D-TOPO reactions without ethanol precipitation did not insert correctly into the pENTR vector. TOPO reactions were carried out for 30 minutes, an extension to the 5 minutes suggested in the protocol.

The successful transformation of cells was confirmed by colony PCR, with vector and gene specific primers (figure 6.3) and DNA sequencing. DNA for all constructs ran at the expected molecular weight of approximately 504 bp on a 1% agarose gel.

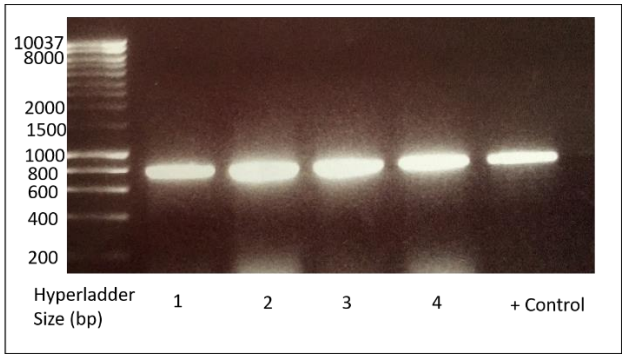

Figure 6.3. *1% agarose gel of colony PCR confirming fusion protein DNA constructs in pENTR vectors. DNA hyperladder was ran alongside 1) GNALeader-GNA-Hv1a, 2) GNALeader-GNA-ModHv1a, 3) GNALeader-Hv1a-GNA, 4) GNALeader-ModHv1a-GNA and a positive control*

Once the sequence is in the pENTR vector the LR recombination reaction was carried out. The pENTR vectors had a kanamycin resistance gene that had to be removed, using restriction digestion using the BspH1 and Sal1 restriction enzymes. The plasmid re-circularised and ethanol precipitation was carried out to make sure the DNA was clean and not contaminated. An extension of the recombination step from 30 minutes to overnight at room temperature was implemented. However even after extensive optimisation of the protocol only 3 of the fusion proteins, GNALeader- GNA-ModHv1a, GNALeader-GNA and GNALeaderGNA-Hv1a, were successfully cloned into the destination vector- pEG201.

Destination vectors containing GNALeader-GNA-ModHv1a were transformed into *Agrobacterium tumefacians* by chemical transformation. They were grown on selective plates and colony PCR confirmed the successful cloning of the vector into *A. tumefacians* ready for infiltration.

6.2.3 Expression of GNALeader-GNA-ModHv1a in *Nicotina benthamiana*

N. benthamiana plants were infiltrated with Agrobacterium GV301 strains, which held resistance to rifampicin and gentamycin. They also contained the pEG201 vector containing GNALeader-GNA-ModHv1a fusion protein which was under the control of the Cam35S promoter. *N. benthamiana* plants were then left for 3 days expressing fusion protein (16:8 hr Light: Dark, 22°C day and 17 °C night).

Protein was then extracted from leaf tissue with two separate leaf samples removed from the plant, macerated, boiled for 5 minutes and centrifuged at 13,000 g for 2 min. Supernatants were collected, with the first of three leaves generating 1120 µl of liquid. 10 and 20 µl of supernatant were used to estimate GNA/ModHv1a concentrations by western blotting (figure 6.4). Samples were loaded onto 17.5% SDS-PAGE gel and after electrophoresis, proteins were transferred to nitrocellulose membranes. These samples were probed with-GNA antibodies and quantified visually against GNA proteins which were used as standard. His-GNA-ModHv1a protein constructs produced in *P. pastoris* were also loaded for comparison and a PEG201 empty vector was loaded as a control.

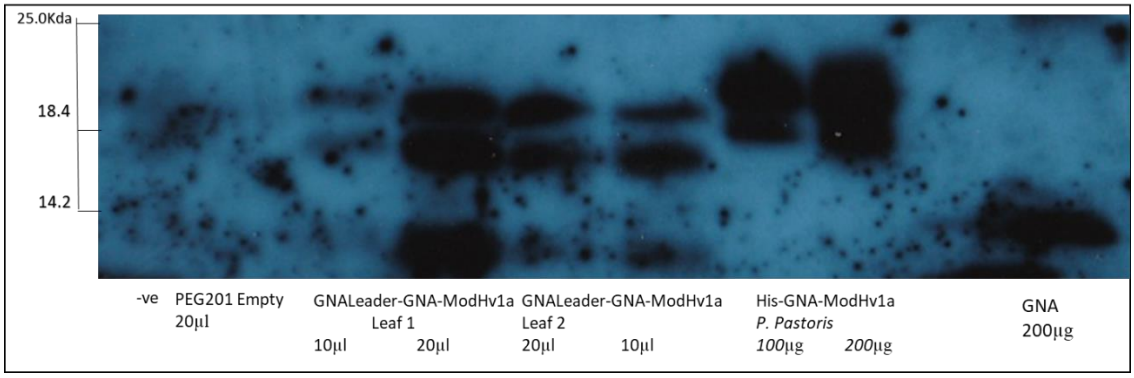

Figure 6.4 Western blot of GNA-Leader-GNA-ModHv1a after being probed with GNA specific antibodies. A negative control, of PEG201 empty vector. GNA Leader-GNA-ModHv1a leaf 1 10 and 20 µl, GNA-Leader-ModHv1a leaf 2 20 and 10 µl. His-GNA-ModHv1a from recombinant expressed P. pastoris is also loaded at 100 and 200 µg. 200 µg of GNA standards is loaded for quantification.

The GNALeader-GNA-ModHv1a protein ran as three distinct bands, the first running at 19 kDa the second at 17 kDa. The two higher molecular weight bands are the intense run at lower masses than that of His-GNA-ModHv1a produced in *P. pastoris* which run at 20k and 18.4 kDa. A third band running at approximately 14 kDa is also visualised when probed by the GNA antibodies in the GNA-Hv1a sample and is not present in the controls or the yeast derived His-GNA-ModHv1a protein.

A Bradford assay of total leaf extract was carried out for both leaves 1 and 2 (data not shown). Visual quantification by western blot analysis using comparisons against GNA standards was used to quantify the amount of intact fusion protein in leaf one was carried out (figure 6.4). Twenty µl of protein extract was shown to contain 200 ng of what was presumed intact fusion protein as compared against GNA standards, 10 µg/µl. Analysis using the same blot to quantify the amount of fusion protein in leaf 2 was not carried out, as the protein loaded was out of range with GNA standards. The expression level of the GNALeader-GNA-ModHv1a protein in *N. benthamiana* was calculated to be 11.3 ng/mg in leaf 1.

6.3 Discussion

6.3.1 GATEWAY™ cloning system

Experiences with the GATEWAY™ cloning system in this chapter proved it to be an inefficient and time-consuming way to clone these fusion protein constructs. This is in contrast to much literature citing the GATEWAY™ system as a highly efficient way of cloning proteins (Katzen, 2007). Even with extensive time spent on protocol optimisation, the cloning was difficult to execute with this slow progress leading to one fusion protein not even being successfully transformed into the pEG201 destination vector. Furthermore, only one protein construct, GNALeader-GNA-ModHv1a, expressed in *Nicotina benthamiana*.

A suite of other cloning methods could have been used to help create constructs in Agrobacterium. These include restriction enzyme cloning, and Type II Assembly (GoldenGate) however were not pursued in this project, on advice that.GATEWAY™ was the best suited and most flexible system.

6.3.2 GNA-ModHv1a expression in *N. benthamiana*

Western blot analysis with GNA specific antibodies of protein supernatant extracted from plants transiently expressing GNAleader-GNA-ModHV1a gene demonstrate that the fusion protein was expressed in *N. benthamiana.* The estimated 1 kDa molecular weight difference compared to His-GNA-ModHv1a produced in yeast could have been accounted by the difference of the 1kDa His tag or differences in glycosylation. The third band is likely to be cleaved processed GNA as it is still reactive to GNA antibodies and runs at a similar molecular weight. More analysis is required to confirm this.

Probing the protein with HA antibodies in a western blot analysis would not have been suitable. Although a HA tag is present in the pEG201 vector it is cleaved during the post translational cleavage of the GNA leader sequence during production of the mature GNA protein in planta. A vector with a HA tag at the C terminus should be sourced and used in future, and a pull-down assay would also then be feasible with the HA tag.

Although both measures are only best estimates, the expression level of the protein in *N. benthamiana* is per gram of leaf tissue is 11.3 ng/mg and is slightly lower than the 25.6 ± 4.1 ng/mg of the Hv1/GNA fusion protein produced by Nakasu et al., 2014.

Two GNA containing cleaved fusion products can be seen for GNA-ModHv1a by western blot analysis. This is in contrast to the comparable protein produced in *P. pastoris* which runs as two tightly spaced bands. Ideally comparison with the other fusion proteins transiently expressed in *N. benthamiana* coupled with comparison to the proteins counterparts in yeast would have been made to elucidate more about the expression of each fusion protein. Due to time constraints further analysis of the protein was not carried out, however future work to characterise this protein would have included glycosylation analysis of the protein bands.

Due to the challenges demonstrated in this thesis it is proposed that future work should continue to work on transient expression, but alongside other projects. . This is required as stable expression of all four fusion protein variants in *Arabidopsis thaliana,* also requires the Gateway cloning system. This would also allow for detached leaf disc bioassay to be carried out using *Myzuz persicae,* a generalist feeder.

6.4 Summary

The work demonstrated that the fusion protein GNA-ModHv1a including a GNA leader sequence at the C terminus can be expressed *in planta* under the control of the constitutive

CaMV 35S promoter. Primary analysis shows that GNA-ModHv1a runs intact with two further cleavage products containing at least partial GNA fragments. Further characterisation of this protein is needed.

Chapter 7. The purification and toxicity assessment of a double toxin fusion protein: ProHvg1-GNA-Hv1a-His (DT1)

7.1 Introduction

When fused to a neurotoxic peptide *Galanthus nivalis* agglutinin; GNA acts as a 'carrier' protein and delivers neurotoxic peptides to the haemolymph where they can reach their site of action (Fitches et al., 2002, 2004, 2012). It is hypothesised that one of the major limiting factors in fusion protein toxicity is the amount of toxin that is able to cross the gut membrane, which has been "carried" by one molecule of GNA (Fitches, 2012).

It is believed that having a neurotoxin fused to both the N and C terminus of GNA, increasing the amount of toxin per one molecule of GNA would increase the toxicity of a fusion protein. ProHvg1-GNA-ModHv1a-His (DT1) is the first "double toxin" fusion protein containing the GNA peptide to be produced and incorporates both Hv1g and Hv1a peptides.

There is currently no published literature on the spider toxin peptide ω-ACTX-Hv1g (Hv1g). Hv1g is a toxic peptide isolated from the Australian funnel web spider, *Hadronyche versuta*, and is similar to Hv1a in that it is another inhibitor cysteine knot type peptide. Therefore, Hv1g possesses many of the same characteristics to Hv1a. It is assumed that the toxin works by interacting by the insect voltage-gated calcium channel, but this has yet to be confirmed. The Hv1g amino-acid sequence also suggests it is identical to the toxin ω-atracotoxin-Arg1 from *Atrax robustus* (Arachno-Server). For use in DT1 the pro region was added to Hv1g to aid folding during recombinant protein production.

The Hv1a toxin-GNA fusion proteins are toxic by injection and orally to *Mamestra brassicae* (Fitches, 2012). The modified version of toxin, Hv1a (ModHv1a) with the K-Q amino acid change which increased the amount of intact fusion protein when produced recombinantly, was used in this double toxin fusion protein construct (Pyati et al., 2014). Data in chapter 5 of this thesis coupled with previous work has shown that GNA containing fusion proteins are still active and retain a similar level of toxicity towards *Acyrthosiphon pisum* regardless of the orientation of the GNA peptide sequence.

There is currently no published data regarding DT1, however preliminary tests of biological activity have shown that the GNA incorporated within the fusion protein is active. This chapter will discuss the purification and quantification of the fusion protein DT1 which has been produced in the yeast strain *Pichia pastoris*. Reactions to determine whether DT1 was glycosylated were carried out and the insecticidal activity of DT1 was assessed by injection into *Mamestra brasiccae.* An LD50 value for DT1 and the individual toxin components against *M. brasiccae* will be determined and compared to LD50's for fusion proteins containing a single toxin. Injection into *M. brasiccae* assessed whether both toxins within DT1 were functionally active and is standard procedure for the laboratory when testing a new fusion protein.

7.2 Results

7.2.1 ProHvg1-GNA-Hv1a-His (DT1) construct

The ProHvg1-GNA-Hv1a-His construct (figure 7.1) was produced and cloned into the pGAPzαB expression vector by Dr Prashant Pyati and Miss Caitlin Willis. A GGGGSAAA linker region was used between both toxins and the GNA as this had been shown to result in the production of almost 100% intact Hv1g/GNA fusion protein when expressed in yeast.

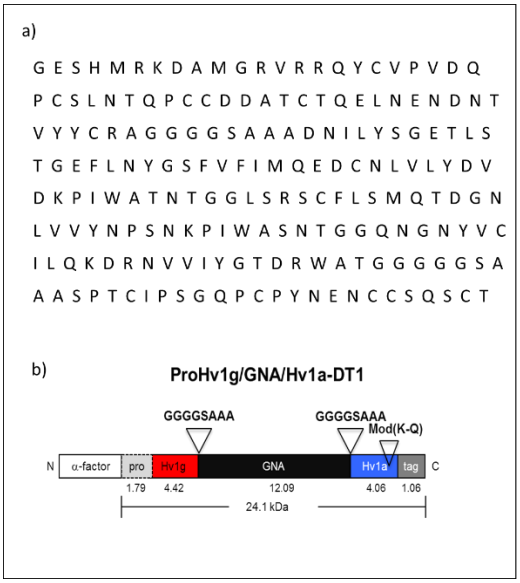

Figure 7.1. a) Predicted amino acid sequence of DT1 construct b) Diagrammatic representation of the DT1 construct. The N terminal alpha factor is highlighted in white, the pro-region in grey, the Hv1g is in red, the black represents GNA, the blue, Hv1a, and the C terminal histidine tag also in grey. The construct has been cloned into the pGAPzαB vector and expressed in Pichia pastoris.

7.2.2 Protein purification from culture supernatant

Bench top fermentation in the Applikon Bioreactor (Fitches, 2012, Pyati 2014) had previously been carried out (Fermenter run #88). Nine hundred mls of neat culture supernatant, which was split into two bottles of 500 ml and 400 ml, was centrifuged and stored at -20°C. The supernatant was thawed fully overnight at 4°C, and 4X binding buffer was added to make up a final concentration of culture supernatant to 1X binding buffer. The supernatant was then filtered, and the protein purified using immobilised nickel affinity chromatography; the protein in solution was loaded onto His-trap nickel columns overnight and then eluted using 200 mM imidazole. The trace for the optical chart recorder for this purification can be seen in figure 7.2.a. After the first elution the supernatant was loaded onto the columns a second time, for a further 4 hours, the trace on the optical chart recorder of protein in the 200mM imidazole elution was slightly reduced demonstrating less protein was eluted than the first load (figure 7.2 b).

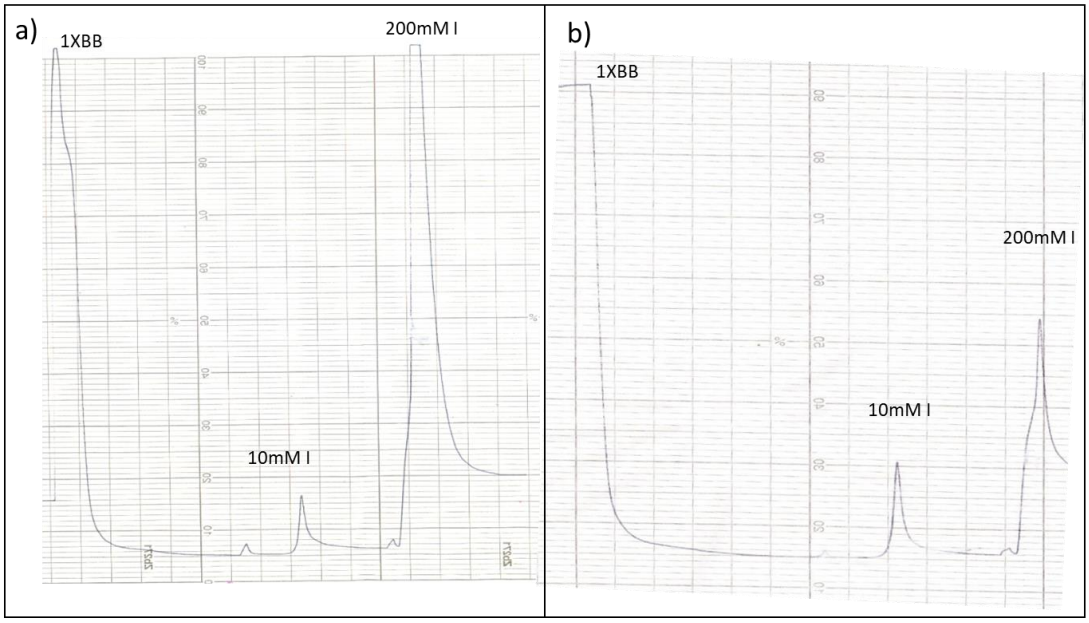

Figure 7.2. a) Chart recorder profile for the first elution of DT1 after the supernatant was loaded onto the columns overnight. 1XBB represents the optical trace recording when a 1X Binding Buffer wash was applied. 10mM I represents the 10mM Imidazole wash and 20mM I, depicts 200mM elution. B) Chart recorder trace of the second elution profile after the protein was loaded for four hours, with a 1XBB, 10mMI and 200mM I peak, note that the 200mM I elution was reduced.

Pooled elution fractions were dialysed in order to remove salts, pigments, cleaved protein fragments and any residual imidazole that may have been left over. Analysis by SDS-PAGE shows that DT1 is only present in 200 mM imidazole wash (figure 7.3). Dialysed protein was then pooled and freeze dried, and lyophilised powder re-suspended at 5 $mgml^{-1}$ in sodium phosphate buffer (SPB). DT1 protein was quantified by SDS-PAGE gel electrophoresis with Coomassie Brilliant Blue (CBB) staining (figure 7.4) against known GNA standards. On the basis that 20µl of the 5 $mgml^{-1}$ solution visually compared to 5µg of GNA standard, the lyophilised powder was quantified to contain 25% intact fusion protein.

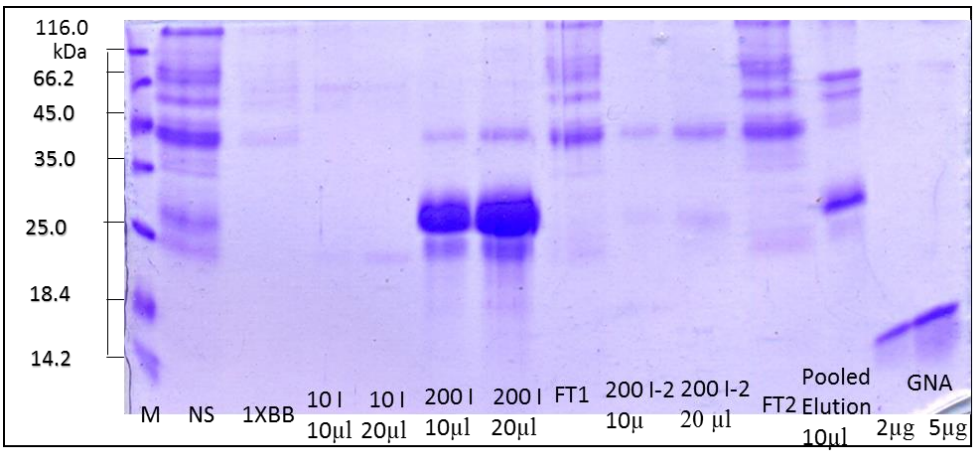

Figure 7.3 SDS-PAGE gel with CBB staining of DT1 elution profile. Samples loaded: Molecular marker (M), 10µl of Neat supernatant (NS), 1X Binding buffer wash 10 µl, 10 and 20 µl of 10mM imidazole wash (10I) and 200mM imidazole elution fraction (200 1), supernatant flow through of both first and second loads (FT1/FT2) alongside 10 µl and 20 µl of the 200mM imidazole elution fractions (200I-2) from the second load were also run on gel. GNA standards of 2 and 5 µg were loaded for comparison and enabled quantification.

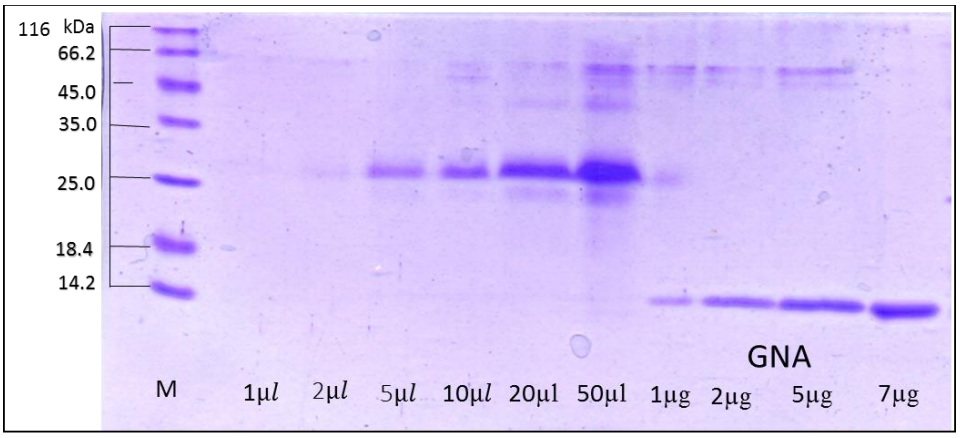

Figure 7.4. *Quantification gel for DT1 lyophilized powder, which was quantified to be 25% protein. Lyophilized powder was re-suspended in SPB at 5mg/ml and loaded onto a 17.5% gel at 1, 2, 5, 10, 20 and 50ul. GNA standards of 1, 2, 5 and 7ug were loaded as standards in order to quantify by visual comparison.*

7.2.3 Analysis of the DT1 protein expressed in *Pichia pastoris*

Re-suspended in SPB, DT1 runs predominantly as a single band on a 17.5% acrylamide SDS-PAGE gel, with a molecular weight of approximately 28.0 kDa. This is higher than the predicted molecular weight of 24 kDa as calculated by the molecular weight calculator, ExPasy. Q-star analysis previously carried out by the laboratory has confirmed that the 28kDa protein band is intact DT1. A fainter second band, which runs at a molecular weight of 24 kDa is only seen when 5 µg or more of intact DT1 is loaded (figure 7.4). Q-star data has confirmed this is DT1 without the pro-region. Molecular weight bands seen at higher molecular weights than this

are assumed to be yeast proteins which contained histidine residues which were able to bind to the nickel His-Trap columns during purification.

7.2.4 Glycosylation of DT1

Periodic acid- Schiff (PAS) staining was undertaken confirming that both proteins bands of DT1 are indeed glycosylated, with bright magenta staining glycoproteins (figure 7.5). The fusion protein PI1a-GNA-His (FP6) was used as a positive control; PI1a is a spider toxin which contains a putative N-glycosylation site (N-X-S/T) at the 35th Asn (Yang et al., 2014). Recombinant GNA was run alongside the samples and does not contain a site for N-linked glycosylation and acted as a negative control.

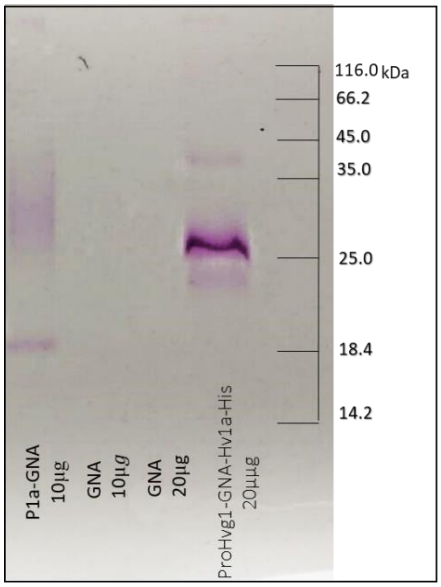

Figure 7.5. *Periodic acid Schiff staining of DT1 protein when loaded at 20µg. 10µg of FP6 was loaded as a positive control. Recombinant GNA (10 and 20µg) acts as a negative control. Magenta staining shows presence of glycosylated protein.*

7.2.5 Injection of DT1 into *Mamestra brassicae* haemolymph to assess DT1's toxicity

In order to assess the biological activity of DT1 *in vivo,* and to establish whether both toxins, Hv1g and ModHv1a in the DT1 fusion protein were active, DT1 was injected directly into the haemolymph of fifth instar *Mamestra brassicae* larvae. Injections with the fusion proteins His-GNA-ModHv1a and ProHv1g-GNA-His were also carried out to aid this comparison. These fusion proteins were chosen as they contained the toxin components present in the DT1 construct.

Stock protein solutions of DT1, His-GNA-ModHv1a and ProHv1g-GNA-His fusion protein were continuously checked by SDS-PAGE gel with CBB staining (figure 7.6) in order to ensure the concentration of stock solutions were accurate. All results showed that they were

comparable to the GNA standards of 2 and 5 µg to which they were compared, one example of this can be seen in Figure 7.6.

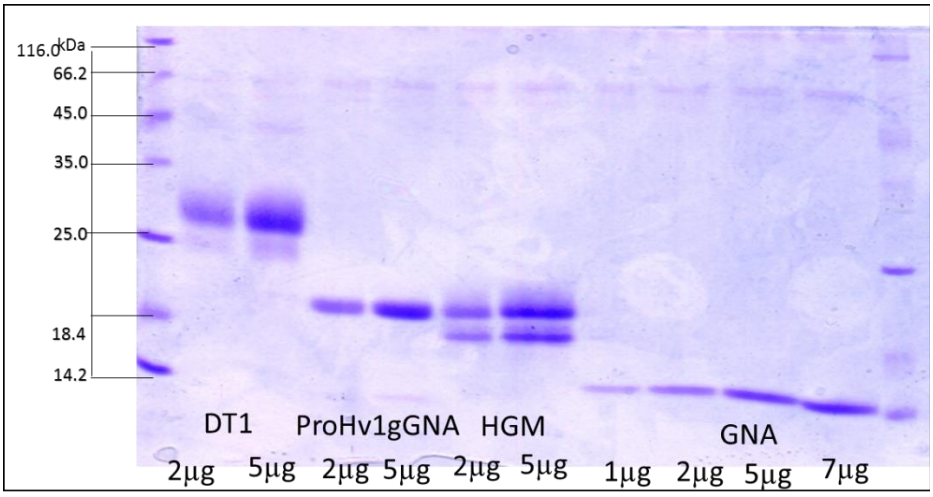

Figure 7.6. DT1, ProHv1g-GNA-His (ProHv1gGNA) and His-GNA-ModHv1a (HGM) re-suspended at 5 mg/ml in SPB and 2 µg and 5 µg were loaded onto an SDS Page gel. 1, 2 and 5 and 7 µg of GNA were loaded onto the gel for visual quantification.

The percentage of Hv1a and Hv1g toxin present in DT1 was calculated based on theoretical molecular weights using ExPASy (Gasteiger et al., 2003). This calculation excluded the pro-region present in both the DT1, and ProHv1g-GNA-His fusion protein as the pro-region is not toxic, but is thought to aid the folding of protein in *Pichia. pastoris.* DT1 without the pro-region was calculated to have a molecular weight of 22.7kDa, 4.1kDa was accounted for by the ModHv1a toxin and 4.4kDa by the Hv1g toxin, 18% and 19% of DT1 respectively. The doses of His-GNA-ModHv1a and ProHv1g-GNA-His injected were designed to match the amount of toxin present in the comparable dose injected of DT1.

The highest dose of intact DT1 injected was 20 µg, this is comparable to higher end of doses of fusion proteins which have previously been tested in this way (Fitches et al., 2004). All *M. brassicae* were fifth instar larvae, and of a similar size and weight; with the mean weight of negative controls being 45.7 mg. *M. brassicae* larvae were anesthetised by being exposed to carbon dioxide for 30 seconds before injection. Injection into the haemolymph with DT1 was followed by a long period of complete paralysis, before another long period of partial paralysis as larvae were allowed to recover on artificial diet; control larvae appeared to recover visibly faster, however this was not quantified.

Post injection, the number of surviving larvae for each condition was recorded every day for four days (figure 7.7, 7.8, 7.9). Larval death occurred over days 1-4 post injection of DT1, although the greatest mortality was seen on days one and two. DT1 displays the highest toxicity when 20 µg is injected, with less than 20% survival. The lowest toxicity was recorded when 1

µg of DT1 was injected with over 60% survival in the *M. brassicae* after 4 days. The survival of larvae injected by DT1 was dose dependent.

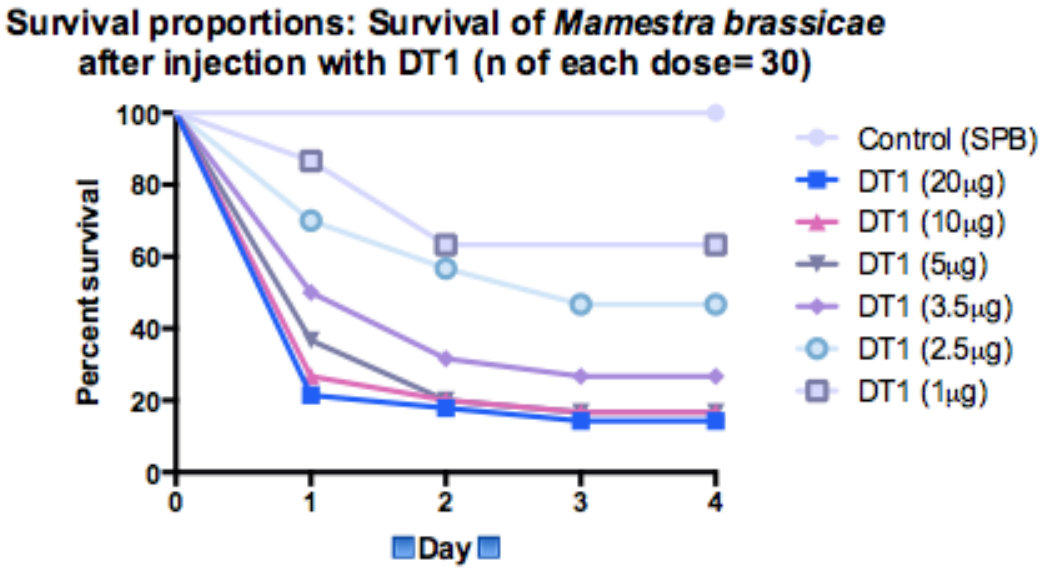

Figure 7.7. *Survival of fifth instar M. brassicae four day days post injection with DT1 at amounts of 1, 2.5, 3.5, 5, 10 and 20µg. Control M. brassicae were injected with 4µl of SPB. Each dose represents data from 30 individuals. P<0.0001*

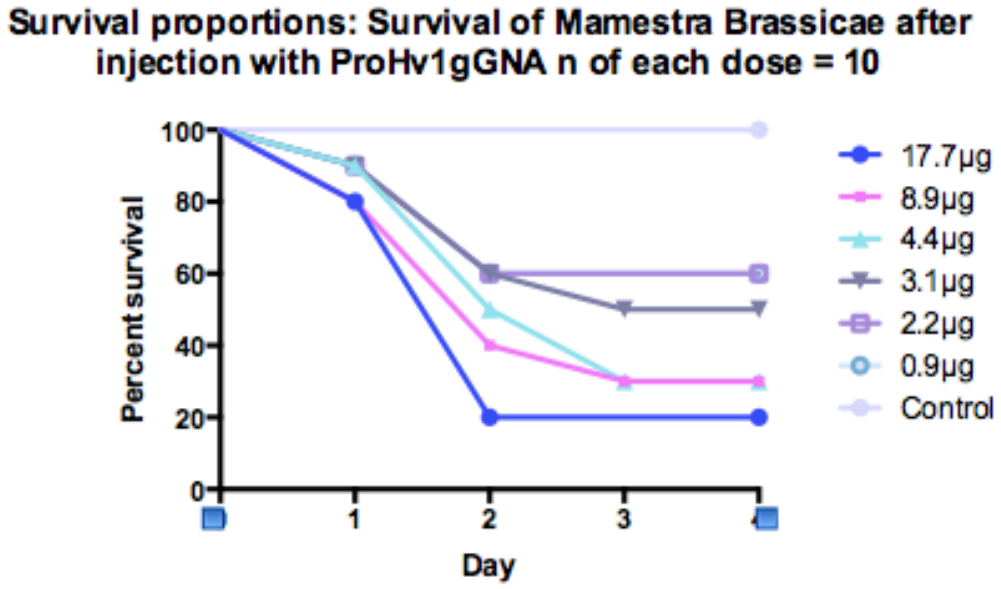

Figure 7.8. *Survival of fifth instar M. brassicae four day days post injection with ProHv1g-GNA-His at doses of 2.5, 3.5, 5, 10 and 20µg. Control M. brassicae were injected with 4µl of SPB. Each dose represents data from 10 individuals. Mantel–Cox log-rank tests P<0.0002*

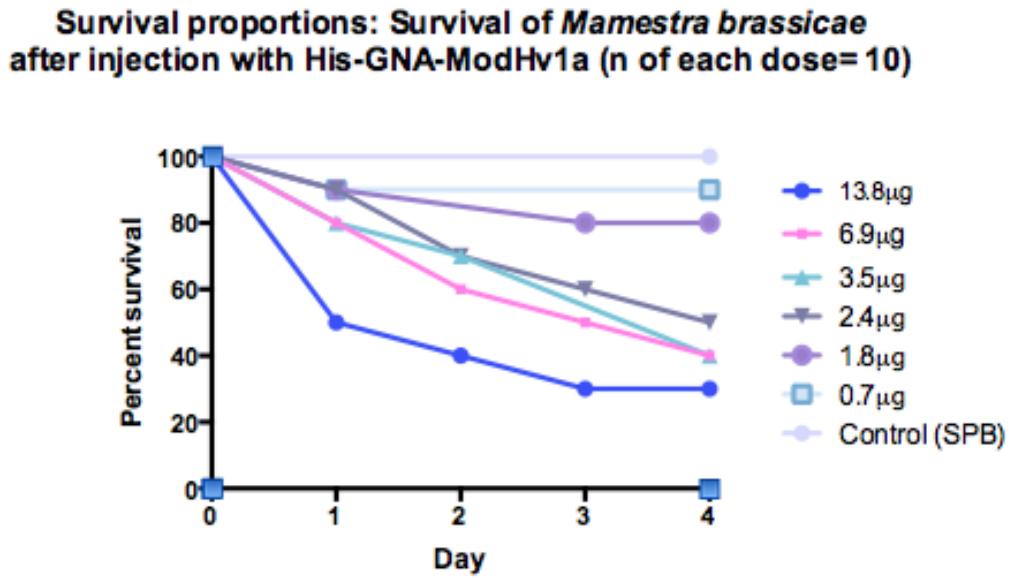

Figure 7.9. *Survival of fifth instar M. brassicae four days post injection with His-GNA-ModHv1a at 0.7, 1.8, 2.4, 3.5, 6.9 and 13.8µg. Control SPB was also injected. Each dose represents data from 10 individuals. Mantel–Cox log-rank tests P<0.0003*

Table 7.1. LD50 values for fusion protein against *M.brassicae* on day 3 and the conferring toxin LD50, suggest both toxins in DT1 are functional and effects are additive.

Fusion Protein	LD50 against *M. brassicae (µg/larvae)*	Toxin dose (µg)
DT1 (ProHv1g-GNA-ModHv1a-His)	2.82	1.06 µg (0.51 µg Hv1a & 0.55 µg Hv1g)
ProHv1g-GNA-His	3.48	0.77 µg Hv1g
His-GNA-ModHv1a	4.08	1.06 µg Hv1a

At comparable doses, the biological activity of DT1 was slightly greater than both proHv1g-GNA-His and His-GNA-ModHv1a (figure 7.8, 7.9). Injected doses of His-GNA-ModHv1a and ProHv1g-GNA-His contained the same amount of the toxin as present in the comparable dose of DT1. Survival data of His-GNA-ModHv1a, show 30% survival at highest dose. Survival data of ProHv1g-GNA-His show 20% survival at the highest dose of 13.8µg of fusion protein. This is greater than the survival of DT1, which showed less than 20% survival.

LD50 values against *M. brassicae* are summarised in table 7.1 and were calculated for DT1, ProHv1g-GNA-His, and His-GNA-ModHv1a using day 3 post injection survival data. The LD50 of DT1 is 2.82 µg and is lower than both ProHv1g-GNA-His and His-GNA-ModHv1a.

The LD50 value for DT1 being 2.82 µg, means that the LD50 value for the combined toxins in DT1 can be calculated as 1.06 µg. The LD50 values for the individual toxins in DT1 can also be calculated as 0.51 µg for ModHv1a and 0.55µg of Hv1g toxin based on molar ratios.

The LD50 value calculated for the His-GNA-ModHv1a fusion protein is 4.08 µg. Therefore, the LD50 for the ModHv1a toxin component is 1.06 µg. The LD50 value calculated for the fusion protein, Pro-Hv1g-GNA is 3.48 µg/ larvae and LD50 for the Hv1g toxin component is therefore 0.77 µg.

Comparing the combined LD50 values of the toxins in DT1 (1.06 µg) against the combined LD50 values of the individual toxin in their respective fusion proteins (1.83 µg), suggests that both toxins in DT1 are functional and effects of having two toxins present in DT1 is additive.

7.3 Discussion

7.3.1 DT1 characterisation

DT1 runs on a SDS-PAGE gel primarily as one intact band, with no significant cleavage products seen. This is in contrast to the single fusion proteins containing GNA and Hv1a, His-GNA-ModHv1a runs as two distinct bands. The use of the longer GGGSSAA linker region between the GNA and two toxins may be the cause of this change in expression pattern, making the protein more resistant to cleavage. The fact that the majority of DT1 is intact when expressed by *Pichia pastoris* is important especially when we consider the feasibility of possible industrial scale up.

7.3.2 DT1 Glycosylation analysis

DT1 lacks any known putative N-glycosylation site with the amino acid sequence (N-X-S/T) where X can be any amino acid, so N-linked glycosylation would not be expected. Previous work on DT1 in the laboratory showed negative results when using a set of deglycosidases specific for all N-linked and some O-linked glycosylation reactions of DT1. However as discussed previously in this thesis (Chapter 4) not all O-linked glycosylated proteins can be removed with the enzymes commercially available. Periodic acid Schiff staining in this chapter shows that DT1 is glycosylated. Both bands in DT1 show positive reactions to Schiff reagent. Furthermore, DT1 runs at approximately 28kDa on a SDS-PAGE gel, yet has a predicted molecular weight of 24.1 kDa, it would seem unlikely that no glycosylation is present.

7.3.3 Evaluating the toxicity of DT1

DT1 is the first fusion protein produced to contain two toxin components fused to a GNA peptide, therefore evaluating its toxicity is essential to distinguish if this was beneficial. The injection of DT1 into the haemolymph of *M. brassicae* larvae resulted in a significant decrease in larvae survival compared to 100% survival control buffer injected larvae.

At comparable doses of toxin, the toxicity of DT1 was higher than both Hv1g present in proHv1g/GNA and the modified Hv1a toxin present in GNA/ModHv1a.

The LD50 values of the fusion proteins confirms that both fusion proteins are functionally active in DT1 and that the effects of two toxins within DT1 are most likely to be additive rather than synergistic. Future work of oral feeding assays with *Mamestra. brassicae* or with Hemipteran pests, would be needed to see if the double toxin increases the amount of toxin transport and increases toxicity.

The toxins present in DT1 both target calcium ion channels and this may explain why the effect of the two toxins is additive rather than synergistic. In this regard a fusion protein named DT2 is being produced in characterised in the lab incorporating two separate toxins targeting different ion channels.

Clearly further analysis of the functionality of DT1 is required and should be pursued. Creation of further fusion proteins with two toxins attached to GNA appears to be an avenue which deserves further attention.

7.4 Summary

The DT1 lyophilised powder was purified from neat culture supernatant by use of immobilised Nickel affinity chromatography and visually quantified by SDS-PAGE gels to contain 25% (w/w) protein. Schiff-PAS staining confirmed that DT1 was in fact glycosylated. Injection of *M. brassicae* larvae with DT1 was compared to injections of proHv1g-GNA-His and His-GNA-ModHv1a and a SPB negative control. LD50 values on day 3 confirmed that DT1 fusion protein was more toxic than both Hv1g and ModHv1a alone and that both toxins were active in the protein with the effects likely to be additive.

Chapter 8. General Discussion

8.1 A new class of insecticides for crop protection

It's vitally important that any new class of insecticide overcomes the limitations of pesticides that are currently commercially available and addresses some of the challenges facing the crop protection industry.

Limitations of current control methods include widespread resistance to a range of insecticides (Miller, 2004), indiscriminate targeting of insect species (including beneficial pollinators), and restrictions on the use of certain insecticidal classes (EU legislation 2009/128/EC and 91/414/EEC). These problems are compounded with increasing demands from the public to avoid certain insecticides and there is a clear need to develop insecticides with a mode of action distinct to any in use.

Biopesticides continue to be explored but many proteins, such as plant derived lectins which have shown to be toxic to a number of insect orders, have proved difficult to introduce into agricultural practice as insecticidal control measures.

Lectins are also not as potent as neurotoxic peptides (Fitches et al., 1997) which can be derived from venomous predators that prey on insects such as spiders and scorpions. These venomous peptides are effective at low doses and although they have not yet been extensively characterised, over 1,500 have currently been described on ArachnoServer as of 2018 (www.archanoserver.org, 2018).

The ω-ACTX-Hv1a (Hv1a) toxin from the Blue mountain funnel web spider, *Hadronyche versuta*, is highly insecticidal and offers considerable potential for use as an insecticide (Windley et al., 2012).

8. 2 Mode of action of venom derived neurotoxins.

Most venom derived peptides exert their effects by interfering with ion channel function via voltage-gated sodium channels in insects' nerve cells, whilst other peptides target calcium or potassium channels. The high specificity of neurotoxins towards particular ion channels, as well as the large potential untapped suite of venomous peptides, highlights their potential as crop protection agents.

Hv1a is a potent antagonist of voltage gated calcium channels (CaV) specific to insects (Tedford et al., 2001). Insects contain a relatively small number of genes encoding for CaV that appear to be essential for function and this makes them attractive as an alternative to conventional pesticides with a novel mode of action, complementing the modes existing pesticides and if used in tandem may help slow down insect resistance (King, 2007).

Although the primary target site of the chemical insecticidal class, pyrethroids, is voltage-gated sodium channels of insect nerves, they are also known to disrupt a variety of voltage gated ligand channels. It is also likely that the unspecific effect pyrethroids exert on many non-target organisms is via target sites other than the voltage-gated sodium channel (Soderlund et al., 2002).

Many spider venoms are dominated by small disulfide rich peptides, like the inhibitor cysteine knot present in Hv1a. This structure confers an advantage to Hv1a, including resistance to heat degradation as well as being extremely potent against insects at low doses (Herzig and King, 2015). It is therefore clear that these venomous peptides offer a large potential suite of new toxins to target insect species.

8.3 GNA as a carrier protein

When venomous toxins are used as insecticides in nature, they are introduced directly into the haemolymph by a bite or sting and thus gain access to neuronal ion channels. However, crop protection agents must be able to cross the insect gut epithelium after oral ingestion. GNA, the snowdrop lectin, is now an established career protein for toxic peptides through the gut and has already been incorporated in a number of fusion proteins (Fitches et al., 2002, 2004, 2010, 2012, Trung et al, 2006, Yang et al., 2014, Pyati et al., 2014). Recently, more light has been shed on the mechanism of transport of GNA across the gut epithelium, where GNA binds preferentially to N-acetylglucosamine (GlcNAc) oligomers in the midgut, further solidifying its important role in insecticide development (Walski et al., 2017).

The novel biopesticide, a fusion protein, containing the spider venom peptide Hv1a from *H. versuta* and GNA was developed by Fitches et al. (2012). Here it was further assessed alongside modified Hv1a/GNA fusion protein constructs generated by Pyati et al, 2014 and a double toxin GNA fusion protein, DT1.

DT1 contains the toxic peptides Hv1a and Hv1g, a HxTx family peptide which target the calcium ion channel and were shown to be toxic to Lepidopteran species, *Mamestra brasiccae* in a dose dependent manner. Both toxic peptides in DT1 were shown to be functional and the effects additive, further supporting the use of these fusion proteins targeting calcium voltage gated ligand channels.

8.4 Fusion proteins in an Integrated Pest Management approach.

It is key that fusion proteins work in an integrated pest management system to be viable as a new major class of insecticides. It's therefore essential that fusion proteins are delivered to insects in the field by multiple means.

Fusion proteins if sprayed onto crops and consumed by insects, would potentially be available to higher trophic levels e.g. arthropods preying on whole insects. Herbivorous insects which are

not pest species will also ingest these fusion proteins which will be able to reach the haemolymph where it will also be taken up by parasitoids. Parasitoids are however likely to be exposed to lower levels of fusion proteins, as they eat target pests rather than feeding directly on leaves sprayed by fusion protein, which is subsequently broken down by the host insect. In fact the parasitoid *Eulophus pennicornis* was not effected when subjected to GNA or the Hv1a/GNA fusion protein (Wakefield et al., 2010; Nakasu, 2014).

The Hv1a/GNA fusion protein has been proven non-toxic to the common honey bee, *Apis mellifera* (Nakasu et al., 2014) unlike other chemical control insecticides, most notably neonicotinoids. Additional safety tests should be carried out to on the Hv1a/GNA class of fusion proteins to confirm it is not harmful to other beneficial insect species.

Aphids feed directly on phloem sap and so to be effective towards this class of insects, the expression of fusion protein in planta is critical. Genetically modified insecticidal plants containing Hv1a/GNA are thus required and here it was shown that it is possible to express an Hv1a/GNA fusion protein in tobacco. Using the GATEWAY cloning system, GNA-ModHv1a was successfully expressed in tobacco. Further work *in planta* should continue to characterize the expression of Hv1a/GNA fusion proteins, which have been successfully expressed (Nakasu et al., 2014) including GNA-ModHv1a and it would be ideal to transform crop plants, with GNA/Hv1a fusion proteins.

Using a high dose/ refuge approach, which is already in use for Bt- expressing plants, might help reduce insect resistance of transgenic plants expressing Hv1a/ GNA.

8.5 Challenges facing recombinant fusion protein commercialisation for insecticides.

The application of biopesticides through sprays is important for integrated pest management strategies.. The use of yeast strains for recombinant protein technology is well established in the pharmaceutical industry, with the spectrum of organisms exploited to be used for recombinant proteins increasing in recent years with around 400 protein drugs are made using recombinant colonies (Sanchez-Garcia et al., 2016). However generating insecticides by recombinant protein for agricultural products remains relatively novel.

In order to maximise the benefit of recombinant fusion proteins, and to produce GNA based fusion proteins at economically viable costs on commercial scale, optimisation of protein production is ongoing (Pyati et al., 2014). Here the expression levels of His-GNA-Hv1a and recovery method were found to be key for maximising the amount of intact fusion protein present in lyophilised powder.

In this thesis the expression profiles of His-GNA-Hv1a, His-GNA-ModHv1a, Hv1a-GNA-His, ModHv1a-GNA-His in *P. pastoris* were further described. Increasing the percentage of intact

fusion protein generated from *P.pastoris,* and reducing cleavage following ingestion by insects will be vital in attempts for commercialisation.

8.6 Alternative approaches to bioinsecticides

How effective fusion proteins are in the field against a broad range of pest species is still unknown. It may be that single transgene expression is not enough and stacking a GNA based fusion protein with Bt toxins should also be explored.

The US biopesticide company Vestaron have done just this, pyramiding Bt toxins with another spider venom peptide very similar to Hv1g, ω-atracotoxin-HxTx. HxTx has been isolated from the venom of *H. versuta* and differs from Hv1g by just 3 residues and Vestaron have produced a new peptide- based family of bioinsectides SPEAR^{TM}, GS-omega-Hxtx-Hv1a – trade-marked as Versitude. Spider-venom derived peptides have now been recognised as a commercial venture, designed to control a number of pests and thus fronts a major new class of sprayable insecticides.

Another option is to produce a double fusion protein, similar to DT1 but targeting multiple insect channels, such a fusion protein is currently being developed and characterised. There is still a large catalogue of potential toxins that have the potential to be order specific that have not yet been extensively studied (Escoubas et al., 2006). Utilising this multiple toxin fusion protein approach is an exciting new direction of research in this field.

It is vital that alternative approaches towards insect resistance in crop protection must continue to be considered including the use of RNA interference (RNAi), where double stranded RNA ingested through expression in transgenic plants or through artificial diet interferes with expression of its target genes. This highly specific approach 'knocks out' the target gene and can be tailored to specific insects therefore avoiding non-target insect species.

8.7 Future work

The inhibitor cysteine knot class of peptides can provide challenges for recombinant expression, but fusion protein production using *Pichia pasto*ris remains an essential expression system for producing large quantities of GNA-spider peptide fusion proteins. Optimising the conditions for purification of the Hv1a/GNA fusion proteins remains important for effective lab work up and, as found in this thesis, alternatives including the use of slow buffer exchange are labour intensive and should be avoided unless absolutely necessary. Future work considering the co-expression or addition of proteinase inhibitors during fusion protein production may help reduce cleavage from restriction enzymes in *P. pastoris*.

One of the main challenges facing economically viable fusion protein production for industry requires enhanced intact fusion protein expression levels. One way to produce more stable

fusion proteins and increase the ratio of intact to cleaved fusion protein present in lyophilised samples is to introduce a longer flexible linker region present in DT1 to all other Hv1a-GNA containing fusion protein variants. DT1 incorporates a GSAAA linker region. Almost 100% of DT1 expressed recombinantly in *P.pastoris* is intact fusion protein as compared to approximately 50% in samples of Hv1a-GNA; further exploration of this linker region in other fusion protein constructs should be pursued. Modifications to the linker region between Hv1a and GNA should increase protein stability and in turn help prevent the fusion protein being cleaved during expression in plants or by ingestion in target. Further GNA based fusion proteins with more than one toxin should be tested and developed, ideally targeting different neurotoxic channels.

This thesis relied on the visual quantification of SDS-PAGE gels which were estimates relying on human eye. An SDS-PAGE gel quantification software is in development; yet was not suitable for use in this thesis, as it was only brought into development halfway through the project and change of method halfway would have proven difficult. This software development will add rigour to the measurements made and use both the number of pixels and pixel intensity of stained GNA samples in scanned images of SDS-PAGE gel and calculates the amount of protein with the same criteria. Further testing of this software should be pursued before it is used routinely.

Conclusion

The results in this thesis show that GNA fusion proteins incorporating spider venom derived toxins are an effective and attractive prospect for use in crop protection products. Expression of these fusion protein constructs recombinantly and *in planta* remains key for an integrated pest management approach. Further improvements to GNA based fusion proteins should continue to be explored to increase the potency of such biopesticides.

References

United Nations, Department of Economic and Social Affairs, Population Division. 2015. World Population Prospects: The 2015 Revision, Key Findings and Advance Tables. Working Paper No. ESA/P/WP.241.

Calzadilla A, Rehdanz K, Betts R, Falloon P, Wiltshire A, Tol RSJ. 2013. Climate change impacts on global agriculture. Climatic Change. Volume 120. Pp 357–374.

Food and Agriculture Organization of the United Nations. 2011. Published in 2011 by the Food and Agriculture Organization of the United Nations Viale delle Terme di Caracalla, 00100 Rome, Italy.

Food and Agriculture Organization of the United Nations. 2009. Published in 2009 by the Food and Agriculture Organization of the United Nations Viale delle Terme di Caracalla, 00100 Rome, Italy.

Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK, Boschung J, Nauels A, Xia Y, Bex V and Midgley PM, (IPCC). 2013. Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, United Kingdom and New York, NY.1535 pp

OECD/FAO. 2012. OECD-FAO Agricultural Outlook 2012. OECD Publishing, Paris. Accessed online at http://dx.doi.org/10.1787/agr_outlook-2012-en. Last accessed n 16/08/2017

Food and Agriculture Organization of the United Nations. Published in 2014 by the Food and Agriculture Organization of the United Nations Viale delle Terme di Caracalla, 00100 Rome, Italy.

Green R, Cornelsen L, Dangour AD, Turner R, Shankar B, Mazzocchi M, Smith RD. 2013. The effect of rising food prices on food consumption: systematic review with meta-regression. BMJ. Volume 346, pp3703.

Schmidhuber J and Tubiello FN. 2007. Global food security under climate change. PNAS. Volume 104, Issue 50, pp 19703–19708.

Solomon S, Qin D, Manning M, Chen Z, Marquis M, Averyt KB, Tignor M and Miller HL. 2007. IPCC. Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996 pp.

Chakrabortya S and Newton AC. 2011. Climate change, plant diseases and food security: an overview. Plant Pathology. Volume 60. pp 2–14.

Zabel F, Putzenlechner B, and Mauser W. 2014. Global Agricultural Land Resources – A High Resolution Suitability Evaluation and Its Perspectives until 2100 under Climate Change Conditions. PLoS One. Volume 9. Issue 9, pe107522.

Hullé M, d'Acier AC, Bankhead-Dromet S , Harrington R. 2010. Aphids in the face of global changes. Compt Rend Biol. Volume 333. pp 497-503.

Bale JSB, Masters GJ, Hodkinson ID, Awmack C, Bezemer TM, Brown VK, Butterfield J, Buse A, Coulson JC, Farrar J, Good JEG, Harrington R, Hartley S, Jones TH, Lindroth RL, Press MC, Symrnioudis I, Watt AD and Whittaker JB. 2002. Herbivory in global climate change research: direct effects of rising temperature on insect herbivores. Global Change Biology. Volume 8. pp 1-16.

Mcvean RIK, Dixon AFG. 2001. The effect of plant drought-stress on populations of the pea aphid *Acyrthosiphon pisum*. Volume 26, Issue 4. pp 440–443.

Woodman JD. 2015. Surviving a flood: effects of inundation period, temperature and embryonic development stage in locust eggs. Bull Entomol Res. Volume 105. Issue 4. pp 441–447.

Stiling P, Cornelissen T. 2007. How does elevated carbon dioxide (CO2) affect plant-herbivore interactions? A field experiment and a meta-analysis of CO2-mediated changes on plant chemistry and herbivore performance. Glob Chan Biol. Volume 13.pp 1823-1842.

United Nations, Department of Economic and Social Affairs, Population Division. 2008. World Population Prospects.

FAO. 2017. The future of food and agriculture – Trends and challenges. Rome.

Hassan R, Scholes R., Ash N, Cassman KG. 2005. Cultivated systems. In Ecosystems and human well-being: current status and trends. Editors. Hassan R., Scholes R., Ash N. pp 745–794.

Schneider A, Friedl MA, Potere D. 2009. A new map of global urban extent from MODIS satellite data. Environ Res Lett. Volume 4, pp 3.

Langeveld JWA, Dixon J, van Keulen H and Quist-Wessel PMF. 2014. Analyzing the effect of biofuel expansion on land use in major producing countries: evidence of increased multiple cropping. Biofuels, Bioproducts and Biorefining. Volume 8, Issue 1, pp 49–58.

Tenenbaum DJ. 2008. Spheres of Influence. Food vs. Fuel: Diversion of Crops Could Cause More Hunger. Environ Health Perspect. Volume 116. Issue 6. pp 254–257.

Zilberman. 2012. The impact of biofuels on commodity food prices: assessment of findings. Am J Agric Econ. Volume 95. pp. 275-281.

Mohr S and Raman. 2013. Lessons from first generation biofuels and implications for the sustainability appraisal of second generation biofuels. Energy Policy. Volume 63, pp 114-122.

Araújo K, Mahajan D, Kerr R and da Silva M. 2017. Global Biofuels at the Crossroads: An Overview of Technical, Policy, and Investment Complexities in the Sustainability of Biofuel Development. Agriculture. Volume 7, Issue 32.

Zhang Z. 2010. Food versus fuel: what do prices tell us. Energy Policy. Volume 38. pp. 445-451.

Murphy SP and Allen LH. 2003. Nutritional Importance of Animal Source Foods. J. Nutr. Volume 133. Issue 11. pp 3932-3935.

Delgado CL. 2003. Rising consumption of meat and milk in developing countries has created a new food revolution. J Nutr. Volume 133 Issue 11. Pp 3907-3910.

Manceron S, Ben-Ari T and Dumas P. 2014. Sciences Protein sources in animal feeds. Feeding proteins to livestock: Global land use and food vs. feed competition. Volume 21. Issue 4. Pp 408.

Oerke EC. 2006. Crop losses to pests. J. Agric. Sci. Volume 144, Issue 31, pp 43.

Gatehouse AMR, Ferry N, Edwards MG, Bell HA. 2011. Insect-resistant biotech crops and their impacts on beneficial arthropods. Philos Trans R Soc Lond B Biol Sci. Volume 366. Issue 1569, pp 1438–1452.

Grube, A., D. Donaldson, T. Kiely, and L. Wu. 2011. Pesticides industry sales and usage: 2006 andm2007 market estimates. Washington, DC: U.S. Environmental Protection Agency. Accessed online at https://www.epa.gov/sites/production/files/2015-10/documents/market_estimates2007.pdf. Last accessed on 14/11/2017

Cai Q.N, Ma X.M, Zhao X, Cao Y.Z, Yang X.Q. 2009. Effects of host plant resistance on insect pests and its parasitoid: a case study of wheat-aphid–parasitoid system. Biol Control. Volume 49, pp 134–138.

Labandeira CC. 2013. A paleobiologic perspective on plant-insect interactions. Current Opinion in Plant Biology. Volume 16. Pp 414–421.

Bar-Joseph, M., Garnsey, S.M. and Gonsalves, D. 1979. The Closteroviruses: a distinct group of elongated plant viruses. Adv. Virus Res. Volume 25. Pp 93-16.

Anderson PK, Cunningham AA, Patel NG, Morales FJ, Epstein PR, Daszak P. 2004. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. Trends Ecol. Evol. Volume 19, pp. 535–544.

Vaughan JA, Scheller LF, Wirtz RA, and Azad AF. 1999. Infectivity of *Plasmodium bergheis*porozoites delivered by intravenous inoculation versus mosquito bite: implications for sporozoite vaccine trials. Infect Immun. Volume 67. Issue 8. Pp 4285–9.

Tolle MA. 2009. Mosquito-borne diseases. Curr Probl Pediatr Adolesc Health Care. Volume 39. Issue 4. Pp 97–140.

Olival KJ, Hosseini PR, Zambrana-Torrelio C, Ross N, Bogich TL, Daszak P. 2017. Host and viral traits predict zoonotic spill over from mammals. Nature. Volume 546. Issue 760. pp 646-650.

Mellet MA and Schoeman AS. 2007. Effect of Bt-cotton on chrysopids, ladybird beetles and their prey: aphids and whiteflies. Indian J. Exp. Biol. Volume 45. Pp 554-562.

Blackman R L, and Eastop V F. 2000. Aphids on the world's crops: an identification and information guide. John Wiley & Sons; New York, NY, USA. pp 476.

van Emden H F, and Harrington R.2007. Aphids as Crop Pests. CABI, Cambridge, UK. Pp 717.

von Dohlen C D, Rowe CA, Heie OE. 2006. A test of morphological hypotheses for tribal and subtribal relationships of Aphidinae (Insecta: Hemiptera: Aphididae) using DNA sequences. Mol Phylogenet Evol. Volume 38. pp 316–329.

Yang S, Pyati P, Fitches E, Gatehouse J.A. 2014. A recombinant fusion protein containing a spider toxin specific for the insect voltage-gated sodium ion channel shows oral toxicity towards insects of different orders. Insect Biochem. Mol. Biol. Volume 47, pp 1–11.

Adams D, Wilkinson TL & Douglas AE. 1996. The aphid-bacterial symbiosis: a comparison between pea aphids and blackbean aphids. Entomologia Experimentalis et Applicata. Volume 80. Pp 275-278.

D'Arcy CJ and Domier LL. 2000. Barley yellow dwarf. The Plant Health Instructor. DOI: 10.1094/PHI-I-2000-1103-01

Powell G, Tosh CR, Hardie J. 2006. Host plant selection by aphids: behavioral, evolutionary, and applied perspectives. Annu Rev Entomol. Volume 51. Pp 309-30.

Scoble MJ. 1992. The Lepidoptera: Form, Function and Diversity. Oxford UK: Oxford University Press. 404 p.

Cates R G. 1980. Feeding patterns of monophagous, oligophagous, and polyphagous insect herbivores: The effect of resource abundance and plant chemistry. Oecologia. Volume 46. Issue 1. Pp 22–31.

Pollini A. 2006. Manuale di entomologia applicata. 1a edizione. Milano, Edagricole: 1462 str

Goulson D. 1994. Determination of larval melanization in the moth, *Mamestra brassicae*, and the role of melanin in thermoregulation. Hereditry. pp 471- 479.

Rojas JC, Wyatt TD, Birch MC. 2000. Flight an oviposition behavior toward different host plant species by the cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae). J. Insect Behav. Volume 13. pp. 247-254.

Pelosini P. 1998. La lotta integrata. Terra e vita 24: 76-78.

Corvi F and Nardi S. 1998. Combattere la nottua del cavolfiore.Terra e vita. Volume 47, Issue 76-78.

Johansen, NS. 1997. Influence of temperature on development, fecundity and survival of the cabbage moth *Mamestra brassicae* (Lep., Noctuidae) in relation to the improvement of forecasting and control methods. Journal of applied entomology. Volume 121, pp 81-88.

Fitches EC, Pyati P, King GF, Gatehouse JA. 2012. Fusion to Snowdrop Lectin Magnifies the Oral Activity of Insecticidal ω-Hexatoxin-Hv1a Peptide by Enabling Its Delivery to the Central Nervous System. PLoS One. Volume 7. Issue 6. p.e39389.

Omino, T., Yokoi, S. and Tsuji, H. 1973. Experimental studies on the daytime behaviour of Noctuid larvae, the cabbage armyworm, *Mamestra brassicae*, the tobacco cutworm, *Spodoptera litura* and the black cutworm, *Agrotis ipsilon*. Japanese Journal of Applied Entomology and Zoology. Volume 17. Issue 4, pp .215-22.0

Burov V and Mokrousova E P. 1970. On the regulating role of the population density of insects after the example of *Barathra brassicae* (Lepidoptera, Noctuidae). Entomol Obozr. Volume 49. pp 257–263.

Devetaki M, Vidrih M, Trdan S. Cabbage moth (*Mamestra brassicae*) and bright-line brown-eyes moth (*Mamestra oleracea*) – presentation of the species, their monitoring and control measures.

Guerrieri, Emilio & F & Tremblay E, Pennacchio.. 1993. Flight behaviour of the aphid parasitoid Aphidius ervi (Hymenoptera: Braconidae) in response to plant and host volatiles. European Journal of Entomology. Volume 90.

Transparency Market Research. Crop Protection Chemicals Market - Global Scenario, Trends, Industry Analysis, Size, Share and Forecast 2011 – 2018. Accessed online at http://www.transparencymarketresearch.com/crop-protection-market.html. Last accessed 09/09/2017.

Oerke EC and Dehne HW. 2004. Safeguarding production—losses in major crops and the role of crop protection. Crop Protection. Volume 23, pp 275–285.

ADAS, 2008. Evaluation of the impact on UK agriculture of the proposal for a regulation of the European Parliament and of the council concerning the placing of plant protection products on the market. Agricultural Development and Advisory Services, Wolverhampton.

Eskenazi B, Marks AR, Bradman A, Fenster L, Johnson C, Barr DB, Jewell NP. 2006. In Utero Exposure to Dichlorodiphenyltrichloroethane (DDT) and Dichlorodiphenyldichloroethylene (DDE) and Neurodevelopment Among Young Mexican American Children. Pediatrics. Volume 118. Issue 1.

Delaplane, K.S., A. Dag, R.G. Danka, B.M. Freitas, L.A. Garibaldi, R.M. Goodwin, and J.I. Hormaza. 2013. Standard methods for pollination research with *Apis mellifera*. 2013. In V Dietemann; J D Ellis; P Neumann (Eds) The COLOSS BEEBOOK, Volume II: standard methods for Apis mellifera research. Journal of Apicultural Research. Volume 51. Issue 5

Livadas GA, Georgopoulos G. 1953. Development of resistance to DDT by Anopheles sacharovi in Greece. Bull World Health Organ. Volume 8. Issue 4. Pp 497–511.

Miller GT. 2004. Sustaining the Earth, 6th edn. Pacific Grove, Ca: Thompson Learning, Inc

Bass C, Denholm I, Williamso MS, Nauen R. 2015. The global status of insect resistance to neonicotinoid insecticides. *Pesticide Biochemistry and Physiology.* Volume 121, Pp 78-87

Liu N, Zhu F, Xu Q, Pridgeon JW, Zhang L. 2006. Behavioral change, physiological modification, and metabolic detoxification: Mechanisms of insecticide resistance. Acta Entomol. Sin. Volume 49. Pp 671–679.

Karunaratne, Jayawardena KGI, Hemingway J, Ketterman AJ. 1993. Characterisation of a B-type esterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus.* Biochem J. Volume 294. pp 575-579.

Klein AM, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proc R Soc B. Issue 24. Pp 303-313.

Nicholls CI, and Altieri MA. 2013. Plant biodiversity enhances bees and other insect pollinators in agroecosystems. A review. Agron Sustain Dev. Volume 33, pp 257–274.

Bryden J, Gill RJ, Mitton RAA, Raine NE. & Jansen, VAA. 2013. Chronic sublethal stress causes bee colony failure. Ecology Letters. Volume 16 Pp 1463–1469.

Cresswell JE. 2010. A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. Ecotoxicology. Volume 20. Pp 149–157.

Jepson JE, Brown LA, Sattelle DB. 2006. The actions of the neonicotinoid imidacloprid on cholinergic neurons of Drosophila melanogaster. Invert Neurosci. Volume 6. Issue 1. Pp 33-40.

Harris R, Monitoring of neonicotinoid resistance in Nilaparvata lugens and subsequent management strategies in Asia Pacific. Proceedings of the International Workshop on Ecology and Management of Rice Planthoppers, Hangzhou, China, 16–19 May 2006, Zhejiang University, p. 2 (2006).

Tomizawa M, Casida JE. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. Annu Rev Entomol. Volume 48. Pp 339-64.

Liong PC, Hamzah WP, Murugan V. 1998. Toxicity of some pesticides towards freshwater fishes. Malaysian Agric J. Volume 54. Issue 3. Pp 147–156.

Kay P, and Grayson R. 2013. Using water industry data to assess the metaldehyde pollution problem. Water Environ. J.pp. 1-8.

Buseques R, Kozynchenko O, Whitby RLD, Tennsion SR, Cundya AB. 2014. Phenolic carbon tailored for the removal of polar organic contaminants from water: A solution to the metaldehyde problem? Water Research. Volume 61. Pp 46-56

Aktar W, Sengupta D, and Chowdhury A. 2009. Impact of pesticides use in agriculture: their benefits and hazards. Interdiscip Toxicol. Volume 2. Issue 1. Pp 1–12.

Forget G. 1993. Balancing the need for pesticides with the risk to human health. In: Forget G, Goodman T, de Villiers A. Impact of Pesticide Use on Health in Developing Countries. 1993. IDRC, Ottawa. pp 2.

Nigam SK, Karnik AB, Chattopadhyay P, Lakkad BC, Venkaiah K, Kashyap SK. 1993. Clinical and biochemical investigations to evolve early diagnosis in workers involved in the manufacture of hexachlorocyclohexane. Int Arch Occup Environ Health. Volueme 65. Issue 1 pp 193-196.

Spiewak R. 2001. Pesticides as a cause of occupational skin diseases in farmers. Ann Agric Environ Med. Volume 8. Issue 1. Pp 1-5.

Keifer MC and Firestone J. 2007. Neurotoxicity of pesticides. J Agromedicine. Volume 12. Issue 1. Pp 17-25.

Basil KL, Vakil C, Sanborn M, Cole DC, Kaur JS, and Kerr KJ. 2007. Cancer health effects of pesticides: Systematic review. Can Fam Physician. Volume 53. Issue 10. pp 1704–1711.

Gunnell D, Eddleston M, Phillips MR, Konradsen F. 2007. The global distribution of fatal pesticide self-poisoning: systematic review. BM Public Health. Volume 7. Pp 357.

2013. https://ec.europa.eu/food/sites/food/files/plant/docs/pesticides_sup_nap_uk_en.pdf)

Chandler D, Bailey AS, Tatchell GM, Davidson G, Greaves J and Grant WP. 2012. The development, regulation and use of biopesticides for integrated pest management. Philos Trans R Soc Lond B Biol Sci. Volume 366. Issue 1573. Pp 1987–1998.

Azzouz H, Cherqui A, Campan ED, Rahbé Y, Duport G, Jouanin L, Kaiser L, and Giordanengo P. 2005. Effects of plant protease inhibitors, oryzacystatin I and soybean Bowman-Birk inhibitor, on the aphid *Macrosiphum euphorbiae* (Homoptera, Aphididae) and its parasitoid *Aphelinus abdominalis* (Hymenoptera, Aphelinidae). J Insect Physiol. Volume 51. Issue 1. Pp 75-86.

Mickel CE, Standish J. 1947. Susceptibility of processed soy flour and soy grits in storage to attack by *Tribolium castaneum*. University of Minnesota Agricultural Experimental Station Technical Bulletin. Volume 178. pp 1-20.

Lawrence PK and Koundal KR. 2002. Plant protease inhibitors in control of phytophagous insects. Election J Biotechnol. Volume 5. Issue 1. Pp 93-109.

De Leo F, Bonadé-Bottino M, Ceci LR, Gallerani R, Jouanin L. 2001. Effects of a mustard trypsin inhibitor expressed in different plants on three lepidopteran pests. Insect Biochem Mol Biol. Volume 31. Issue 6-7. Pp 593-602.

Falco MC & Silva-Filho MC. 2003. Expression of soybean proteinase inhibitors in transgenic sugarcane plants: effects on natural defense against *Diatraea saccharalis*. Plant Physiology and Biochemistry. Volume 41. Pp 761–766.

Alfonso-Rubí J, Ortego F, Castañera P, Carbonero P, Díaz I. 2003. Transgenic expression of trypsin inhibitor CMe from barley in indica and japonica rice, confers resistance to the rice weevil *Sitophilus oryzae*. Transgenic Res. Volume 12. Issue 1. Pp 23-31.

Sanahuja G, Banakar R, Twyman RM, Capell T and Christou P. 2011. *Bacillus thuringiensis:* a century of research, development and commercial applications. Plant Biotechnology Journal. Volume 9. Pp 283–300.

Ignoffo CM, Garcia C, Kroha MJ, Fukuda T, Couch TL. 1981. Laboratory tests to evaluate the potential efficacy of Bacillus thuringiensis var. israelensis for use against mosquitoes. Mosq. News. Volume 41. Pp 85–93.James C. 2013. Global status of commercialized biotech GM crops: 2013. Brief No. 46. ISAAA, Ithaca, New York.

Tabashnik BE, Brévault T, Carrière Y. 2013. Insect resistance to Bt crops: lessons from the first billion acres. Nature Biotechnology. Volume 31. Pp. 510

Cao J, Zhao JZ, Tang JD, Shelton AM and Earle ED. 2002. Broccoli plants with pyramided cry1Ac and cry1C Bt genes control diamondback moths resistant to Cry1A and Cry1C proteins. Theor Appl Genet. Volume 105. Pp 258–264.

Zhao JZ, Cao J, Li YX, Collins HL, Roush RT, Earle ED and Shelton AM. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. Volume 21. Pp 1493–1497.

Bravo A, Miranda R, Gómez I, and Soberón M. 2002. Pore formation activity of Cry1Ab toxin from *Bacillus thuringiensis* in an improved membrane vesicle preparation from *Manduca sexta* midgut cell microvilli. Biochimica et Biophysica Acta (BBA) – Biomembranes. Volume 1562, Issues 1–2, 3 May 2002, Pp 63–69.

Zhang X, Candas M, Griko NB, Taussig R, Bulla LA Jr. 2006. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of Bacillus thuringiensis. Proc Natl Acad Sci U S A. Volume 103. Issue 26. Pp 9897-902.

Shimada N, Miyamoto K, Kanda K, Murata H. 2006. *Bacillus thuringiensis* insecticidal Cry1ab toxin does not affect the membrane integrity of the mammalian intestinal epithelial cells: An *in vitro* study. *In vitro* Cell Dev Biol Anim. Volume 42. Issue 1-2. Pp 45-9.

Federici B. 2002. Case study: Bt crops a novel mode of insect control. In Genetically Modified Crops: Assessing Safety, ed. KT Atherton, pp. 164–200. London: Taylor & Francis

Klümper W and Qaim M. 2014. A meta-analysis of the impacts of genetically modified crops. PLoS One. Volume 3. Issue 9. 11 pages.

Jackson RE, Gould F, Bradley JR & Van Duyn J. 2006. Genetic variation for resistance to Bacillus thuringiensis toxins in *Helicoverpa zea* (Lepidoptera: Noctuidae) in eastern North Carolina. J Econ Entomol. Volume 99. Pp 1790–1797.

Brookes G and Barfoot P. 2015. Environmental impacts of genetically modified (GM) crop use 1996-2013: Impacts on pesticide use and carbon emissions. GM Crops Food. Volume 6. Issue 3. Pp 103-133.

Regulation (EC) No 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. Official Journal L. Volume 268 Pp 24 – 28. Accessed online at https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:32003R1830 last accessed on 24/06/2018.

Brandt P. 2003. Overview of the current status of genetically modified plants in Europe as compared to the USA. J Plant Physiol. Volume 160. Issue 7. Pp 735-42.

Lewis WJ, van Lenteren JC, Phatak SC and Tumlinson JH. 1997. A total system approach to sustainable pest management. Proc Natl Acad Sci. Volume 94. Issue 23. pp 12243–12248.

Andow D. 1983. The extent of monoculture and its effects on insect pest populations with particular reference to wheat and cotton. Agriculture, Ecosystems & Environment. Volume 9, Issue 1. Pp 25-35

Pretty J and Bharucha ZP. 2015. Integrated Pest Management for Sustainable Intensification of Agriculture in Asia and Africa. Insects. Volume 6. Issue 1. Pp 152-182.

Wilkins RJ. 2008. Eco-efficient approaches to land management: a case for increased integration of crop and animal production systems. Philos Trans R Soc Lond B Biol Sci.Volume 363. Issue 1491. Pp 517–525.

Carrière Y, Ellers-Kirk C, Sisterson M, Antilla L, Whitlow M, Dennehy TJ, Tabashnik BE. 2003. Long-term regional suppression of pink bollworm by Bacillus thuringiensis cotton. Proc Natl Acad Sci. Volume 100. Issue 4. Pp 1519-23.

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED and Shelton AM. 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nature biotechnology. Volume 21. Issue 12. Pp 1493-1497.

Garrison NL, Rodriguez J, Agnarsson I, Coddington JA, Griswold CE, Hamilton CA, Hedin M, Kocot KM, Ledford JM and Bond JM. 2016. Spider phylogenomics: untangling the Spider Tree of Life. PeerJ. Volume 4, pp 1719.

Windley MJ, Herzig V, Dziemborowicz SA, Hardy MC, King GF, and Nicholson GM. 2012. Spider-Venom Peptides as Bioinsecticides. Toxins. Volume 4. Issue 3. Pp 191–227.

Saez NJ, Senff S, Jensen JE, Er SY, Herzig V, Rash LD and King GF. 2010. Spider- Venom Peptides as Therapeutics. Toxins (Basel). Volume 2. Issue 12. Pp 2851 – 2871.

Platnick NI. 2011. The World Spider Catalog, Version 13.0. World Spider Catalog (2017). World Spider Catalog. Natural History Museum Bern, online at http://wsc.nmbe.ch, version 18.5, accessed on 21/09/2017.

Escoubas P, Diochot S, Corzo G. 2000. Structure and pharmacology of spider venom neurotoxins. Biochimie. Volume 82. Issue 9-10. Pp 893-907.

King GF and Hardy MC. 2013. Spider-venom peptides: Structure, pharmacology, and potential for control of insect pests. Annu. Rev. Entomol. Volume 58, pp 475–49.

King GF. 2011. Venoms as a platform for human drugs: translating toxins into therapeutics. Expert Opin Biol Ther. Volume 11. pp 1469-1484.

Wang X, Smith R, Fletcher JI, Wilson H, Wood CJ, Howden ME, King GF. 1999. Structure-function studies of omega-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. Eur J Biochem. Volume 264, Issue 2, pp 488-94.

Atkinson RK, Howden MEH, Tyler MI and Vonarx, EJ. 1993. Insecticidal toxins derived from funnel web spider (Atrax or Hadronyche) spiders. International Patent Application PCT/AU93/00039 (WO 93/15108).

King G F. 2007. Modulation of insect Cav channels by peptidic spider toxins. Toxicon. Volume 49. Issue 4. Pp 513-530. d

Tedford HW, Sollod BL, Maggio F, King GF. 2004. Spider venoms. Australian funnel-web spiders: master insecticide chemists. Toxicon. Volume 43, Issue 5, pp 601–618.

Tedford HW, Fletcher J, King GF. 2001. .Functional significance of the beta hairpin in the insecticidal neurotoxin omega-atracotoxin-Hv1a. J Biol Chem. Volume 276. Issue 28. Pp 26568-76

Herzig V and King GF. 2015. The Cystine Knot Is Responsible for the Exceptional Stability of the Insecticidal Spider Toxin ω-Hexatoxin-Hv1a. Toxins. Volume 7, Issue 10, pp 4366-80.

Mukherjee AK, Sollod BL, Wikel SK, King GF. 2006. Orally active acaricidal peptide toxins from spider venom. Toxicon. Volume 47. Issue 2. Pp 182- 187.

Nakasu EYT, Edwards MG, Fitches E, Gatehouse JA and Gatehouse AMR. 2014. Transgenic plants expressing ω-ACTX-Hv1a and snowdrop lectin (GNA) fusion protein show enhanced resistance to aphids. Front Plant Sci. Volume 5, pp 673.

Back E. J. (2011). Insecticidal Fusion Proteins for the Control of Coleopteran Pests. Ph.D. thesis, Durham University, Durham.

Wood DLA, Miljenović T, Cai S, Raven RJ, Kaas Q, Escoubas P, Herzig V, Wilson D, and King GF. 2009. ArachnoServer: a database of protein toxins from spiders. BMC Genomics Volume 10. Pp 375

Macedo MLR, Oliveira CFR and Oliveira CT. 2015. Insecticidal Activity of Plant Lectins and Potential Application in Crop Protection. Molecules. Volume 20, pp 2014-2033.

Peumans WJ and Van Damme EJM. 1995. Lectins as plant defense proteins. Plant Physiol Volume 109, pp 347–352.

Van Damme EJM, Peumans WJ, Barre A, Rouge P. 1998. Plant lectins: A composite of several distinct families of structurally and evolutionary related proteins with diverse biological roles. Crit. Rev. Plant Sci. Volume 17, pp 575–692.

Lam SK and Ng TB. 2011. Lectins: production and practical applications. Appl Microbiol Biotechnol. Volume 89. Issue 1. Pp 45-55.

Regente M, Taveira GB, Pinedo M, Elizalde MM, Ticchi AJ, Diz MS, Carvalho AO, de la Canal L, Gomes VM. 2014. A sunflower lectin with antifungal properties and putative medical mycology applications. Curr Microbiol. Volume 69. Issue 1. Pp 88-95

Van Damme EJM, Allen AK, Peumans WJ. 1987. Isolation and characterisation of a lectin with exclusive specificity towards mannose from snowdrop (Galanthus nivalis) bulbs. FEBS Letters. Volume 215, Issue 1, 4 May 1987, Pages 140-144.

Van Damme E.J.M, Kaku H, Perini F, Goldstein I.J, Peeters B, Yagi F, Decock B, Peumans W.J. 1991. Biosynthesis, primary structure and molecular cloning of snowdrop (*Galanthus nivalis*) lectin. Eur. J. Biochem. Volume 202. pp 23-30.

Fouquaert E, Hanton SL, Brandizzi F, Peumans WJ, Van Damme ELM. 2007. Localization and Topogenesis Studies of Cytoplasmic and Vacuolar Homologs of the *Galanthus nivalis agglutinin*. Plant and Cell Physiology, Volume 48, Issue 7, Pp 1010–1021.

Hester G, Kaku H, Goldstein I.J and Wright C.S. 1995. Structure of mannose-specific snowdrop (*Galanthus nivalis*) lectin is representative of a new plant lectin family. Nature Structural Biology. Volume 2, pp 472 – 479.

Van Damme, Nakamura-Tsurua S, Smith DF, Ongenaert M, Winter HC, Rouge P, Godlstein IJ. 2007. Phylogenetic and specificity studies of two-domain GNA-related lectins: generation of multispecificity through domain duplication and divergent evolution. Els J. Biochem. J. Volume 404, pp 51–61

Rao KV, Rathore KS, Hodges TK, Fu X, Stoger E, Sudhakar D, Williams S, Christou P, Bharathi M, Bown DP, Powell KS, Spence J, Gatehouse AM, Gatehouse JA. 1998. Expression of snowdrop lectin (GNA) in transgenic rice plants confers resistance to rice brown planthopper. Plant J. Volume 15. Issue 4. Pp 469-77.

Fitches, E., Gatehouse, A.M. and Gatehouse JA. 1997. Effects of snowdrop lectin (GNA) delivered via artificial diet and transgenic plants on the development of tomato moth (*Lacanobia oleracea*) larvae in laboratory and glasshouse trials. Journal of Insect Physiology. Volume 43. Issue 8. Pp727-739.

Nutt KA, Allsopp PG, McGhie TK, Shepherd KM, Joyce PA, Taylo GO, McQualter RB Smith GR and Hogarth DM. 1999. Transgenic sugarcane with increased resistance to canegrubs. Proceedings of the 1999 Conference of the Australian Society of Sugarcane Technologists. Australia. Pp 171-176.

Gatehouse A.M.R, Powell K.S, Peumans W.J, Van Damme E.J.M & Gatehouse J.A. 1995. Insecticidal properties of plant lectins: their potential in plant protection. Lectins: Biomedical Perspectives. Taylor & Francis Ltd U.K. pp. 35–57.

Romeis J, Babendreier D, Wäckers F.L. 2003. Consumption of snowdrop lectin (Galanthus nivalisagglutinin) causes direct effects on adult parasitic wasps. Oecologia. Volume 134, Issue 4, pp 528-36.

Pusztai A. 1991. Plant Lectins. Chemistry and Pharmacology of Natural Products. 263 pages.

Poulsen, Kroghsbo S, Schrøder M, Wilcks A, Jacobsen H, Miller A, Frenzel T, Danier J, Rychlik M, Shu Q, Emami K, Sudhakar D, Gatehouse A, Engel KH, Knudsen I. 2007. A 90-day safety study in Wistar rats fed genetically modified rice expressing snowdrop lectin *Galanthus nivalis* (GNA). Food Chem Toxicol. Volume 45. Issue 3. Pp 350-63.

Fitches E and Gatehouse JA. 1998. A comparison of the short and long term effects of insecticidal lectins on the activities of soluble and brush border enzymes of tomato moth larvae (*Lacanobia oleracea*). J Insect Physiol. Volume 44. Issue 12. Pp 1213-1224.

Fitches E, Woodhouse SD, Edwards JP, Gatehouse JA. 2001. *In vitro* and *in vivo* binding of snowdrop (*Galanthus nivalis* agglutinin; GNA) and jackbean (*Canavalia ensiformis*; Con A) lectins within tomato moth (*Lacanobia oleracea*) larvae; mechanisms of insecticidal action.J Insect Physiol. Volume 47. Issue 7. Pp 777-787.

Malone LA, Gatehouse AMR, Barratt BIP. 2008. Beyond Bt: alternative strategies for insect-resistant genetically modified crops. Integration of Insect-Resistant Genetically Modified Crops within IPM Programs. Springer. Pp 357-417.

Li Y, Romeis J. 2009. Impact of snowdrop lectin (Galanthus nivalis agglutinin; GNA) on adults of the green lacewing, *Chrysoperla carnea.* Journal of Insect Physiology. Volume 55. Issue 2. Pp 136-143.

Lawo NC, Romeis J. 2008. Assessing the utilization of a carbohydrate food source and the impact of insecticidal proteins on larvae of the green lacewing, *Chrysoperla carnea*. Biological Control, Volume 44. Pp 389-398.

Fitches E, Edwards MG, Mee C, Grishin E, Gatehouse AM, Edwards JP, Gatehouse JA. 2004. Fusion proteins containing insect-specific toxins as pest control agents: snowdrop lectin delivers fused insecticidal spider venom toxin to insect haemolymph following oral ingestion. J Insect Physiol. Volume 50. Issue 1. Pp 61-71.

Hogervorst PA, Ferry N, Gatehouse AM, Wäckers FL and Romeis J. 2006. Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. Journal of insect physiology. Volume 52. Issue 6. Pp 614-624.

Powell KS, SPance J, Bharath M, Gatehouse JA, Gatehouse AMR. 1998. Immunohistochemical and development studies to elucidate the mechanism of action of the snowdrop lectin on the rice brown planthopper, *Nilaparvata lugens* (Stal). J Insect Physiol. Volume 67. Pp 529-539.

Walski T, De Schutter K, Cappelle1 K, Van Damme EJM and Smagghe G. 2017. Distribution of Glycan Motifs at the Surface of Midgut Cells in the Cotton Leafworm (*Spodoptera littoralis*)

Demonstrated by Lectin Binding. Front Physiol. Volume 8. Accessed online at https://www.frontiersin.org/articles/10.3389/fphys.2017.01020/full. Last accessed on 22/12/17

Van Emden H.F. 1999. Transgenic host plant resistance to insects—some reservations. Ann Entomol Soc Am. Volume 92, pp788–797.

Beck A and Reichert JM. 2011. Therapeutic Fc-fusion proteins and peptides as successful alternatives to antibodies Sep-Oct. Volume 3. Issue 5. Pp 415-6.

Pyati P, Fitches E, Gatehouse J.A . 2014. Optimising expression of the recombinant fusion protein biopesticide ω-hexatoxin-Hv1a/GNA in *Pichia pastoris*: sequence modifications and a simple method for the generation of multi-copy strains. Journal of Industrial Microbiology & Biotechnology. Volume 41, Issue 8, pp 1237–1247.

Bown, D.P., Wilkinson, H.S. and Gatehouse, J.A., 1997. Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, Helicoverpa armigera, are members of complex multigene families. Insect biochemistry and molecular biology, 27(7), pp.625-638.

Bretthauer RK, Castellino FJ. 1999. Glycosylation of *Pichia pastoris* -derived proteins. Volume 30, Issue 3. Pp 193–200.

Laemmli. 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature. 1970. Volume 15, Issue 227, pp 680

Shapiro AL, Viñuela E, Maizel Jr JV. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochemical and Biophysical Research Communications. Volume 28, Issue 5, pp 815-820.

Higgins DR and Cregg JM. 1998. Introduction of *Pichia pastoris*. Methods Mol Biol. Volume 103. Pp 1 -15

Tschopp JF, Sverlow G, Kosson R, Craig W and Grinna L. 1987. High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. Nature Biotechnology, Volume 5. Issue 12. Pp 1305-130.

Birnboim HC, and Doly J.1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. Volume 7, Issue 6, pp 1513–23.

Kunkel H. 1976. Membrane feeding systems in aphid research, in Aphids as Virus Vectors eds Harris KF., Maramorosch K., editors London: Academic Press. Pp 311–337.

Prosser WA and Douglas AE. 1992. A test of the hypotheses that nitrogen is upgraded and recycled in an aphid (*Acyrthosiphon pisum*) symbiosis. J. Insect Physiol. Volume 38. Pp 93–99.

Bradford MM. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem. Volume 7. Issue 72. Pp 248-54.

Packer NH, Ball MS, Devine PL, Patton WF and Walker JM. 2002. Detection of Glycoproteins in Gels and Blots. The Protein Protocols Handbook. pp 761-772

Kurtzman CP. 2009. Biotechnological strains of Komagataella (Pichia pastoris) are Komagataella phaffii as determined from multigene sequence analysis. J Ind Microbiol Biotechnoly. Volume 36. Issue 11. Pp 1435-8.

Lie P, Anumanthan A, Gao XG, Ilangovan K, Suzara VV, Düzgüneş N, Renugopalakrishnan V. 2007. Expression of recombinant proteins in *Pichia pastoris.* Appl Biochem Biotechnol. Volume 142. Issue 2. Pp 105-24.

Ahmed M, Hirz M, Pichler H, and Schwab H. 2014. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol. Volume 98. Issue 12, pp 5301–5317.

Cereghino JL and Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev. Volume 24. Issue 1. Pp 45-66.
Morrell, B., 2016. Insecticidal fusion proteins: How does the orientation of the toxin to the carrier affect toxicity to insects? Masters thesis. University of Durham

Lin-Cereghino GP, Stark CM, Kim D, Chang J, Shaheen N, Poerwanto H, Agari K, Moua P, Low LK, Tran N, Huang AD, Nattestad M, Oshiro KT, Chang JW, Chavan A, Tsai JW, and Lin-Cereghino J. 2013. The effect of α-mating factor secretion signal mutations on recombinant protein expression in Pichia pastoris. Gene. Volume 519. Issue 2. Pp 311-317.

Carson M, Johnson DH, McDonald H, Brouillette C and Delucas. 2007. His-tag impact on structure. Acta Crystallogr D Biol Crystallogr. Volume 63. Issue 3. Pp 295-301.

Bornhorst JA.and Falke JJ. 2000. Purification of Proteins Using Polyhistidine Affinity Tags. Methods Enzy mol.Volume 326. Pp 245-254.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. 2005. Heterologous protein production using the *Pichia pastoris* expression system. Yeast. Volume 22. Pp 249–270

Bourbonnais Y, Bolin, D and Shields D (1988) Secretion of Somatostatin by Saccharomyces cerevisiae. The journal of biological chemistry. Vol. 263, No. 30, Issue of October 25, pp. 15342-1534.

Khoury GA, Baliban RC & Floudas CA. 2011. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. Scientific Reports. Volume 90. Issue 2011.

Goettig P. 2016. Effects of Glycosylation on the Enzymatic Activity and Mechanisms of Proteases. Int J Mol Sci. Volume 17. Issue 12. Pp 1969.

Doerner KC and White BA. 1990. Detection of glycoproteins separated by nondenaturing polyacrylamide gel electrophoresis using the periodic acid-Schiff stain. Anal Biochem. Volume 187. Issue 1. Pp 147-150.

Brady PN, Macnaughtan MA. 2015. Evaluation of Colorimetric Assays for Analyzing Reductively Methylated Proteins: Biases and Mechanistic Insights. Analytical biochemistry. 2015. Volume 491. Pp 43-51.

Katzen F. 2007. Gateway® recombinational cloning: a biological operating system. Expert Opinion on Drug Discovery Volume 2, Issue 4.

Fitches E, Audsley N, Gatehouse JA, Edwards JP. 2002. Fusion proteins containing neuropeptides as novel insect contol agents: snowdrop lectin delivers fused allatostatin to insect haemolymph following oral ingestion. Insect Biochem Mol Biol. Volume 32. Issue 12. Pp 165-61.

Yang, S., Fitches, E., Pyati, P. & Gatehouse, J.A. (2015). Effect of insecticidal fusion proteins containing spider toxins targeting sodium and calcium ion channels on pyrethroid-resistant strains of peach-potato aphid (*Myzus persicae*). Pest Management Science. Volume 71. Issue 7. Pp 951-956.

Devonshire AL, Field LM, Foster JP, Moores GD, Williamson MS and Blackman RL. 1998. The evolution of insecticide resistance in the peach-potato aphid, *Myzus persicae*. Philos Trans R Soc Lond. B Biol. Sci. Volume 353. Pp 1677–1684.

Chougule NP and Bonning BC. 2012. Toxins for Transgenic Resistance to Hemipteran Pests. Toxins (Basel). Volume 4. Issue 6. Pp 405–429.

Fitches EC, Bell HA, Powell ME, Back E, Sargiotti C, Weaver RJ, Gatehouse JA. 2010. Insecticidal activity of scorpion toxin (ButaIT) and snowdrop lectin (GNA) containing fusion proteins towards pest species of different orders. Pest Manag Sci. Volume 66. Issue 1. Pp 74-83.

Lopez, PL, Soberón M, Bravo A. 2013. Bacillus thuringiensis insecticidal three-domain Cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbiology Reviews, Volume 37, Issue 1, Pp 3–22.

Published: 01 January 2013 Article historyPorcar M, Grenier AM, Federici B and Rahbé Y. 2009. Effects of Bacillus thuringiensis δ-Endotoxins on the Pea Aphid (*Acyrthosiphon pisum)*. Appl. Environ. Microbiol. Volume 75. Issue 14. pp 4897-4900.

Brévault T, Heuberger S, Zhang M, Ellers-Kirk C, Ni X, Masson L, Li X, Tabashnik BE, Carrière Y. 2013. Potential shortfall of pyramided transgenic cotton for insect resistance management. Proc Natl Acad Sci. Volume 110. Issue 15. Pp 5806-11.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel R.D, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. Volume 31, pp 3784-3788.

Sanchez-Garcia L, Martín L, Mangues R, Ferrer-Miralles N, Vázquez E, and Villaverde A. 2016. Recombinant pharmaceuticals from microbial cells: a 2015 update. Microb Cell Fact. Volume 15. Issue 33.

Tritkova M, Wikmark OG, Zemp N, Widmer A and Hilbeck A, Alvarez ML. 2015. Transgene Expression and Bt Protein Content in Transgenic Bt Maize (MON810) under Optimal and Stressful Environmental Conditions. PLoS One.Volume 10. Issue 4.

www.arachnoserver.org. 2018. Accessed online at www.arachnoserver.org. Last accessed on 08/08/2018

Soderlund DM, Clark JM, Sheets LP, Mullin LS, Piccirillo VJ, Sargent D, Stevens JT, Weiner ML. 2002. Mechanisms of pyrethroid toxicity: implications for cumulative risk assessment. Toxicology. Volume 171.Pp3–59.