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New environmentally-friendly technologies for slug control based on orally-delivered fusion proteins containing specific molluscicidal toxins

by

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1. ABSTRACT

The aim of this project was to investigate the potential for a novel technology, originally conceived and developed for the control of insect pests, to be extended for the control of mollusc crop pests, with focus on the grey field slug *Deroceras reticulatum*.

This technology allows naturally occurring proteins, which have low, or no, toxicity when delivered orally to be converted into effective and orally active pesticides. The approach, patented by the academic partners, uses genes encoding fusion proteins that contain a plant-derived protein carrier (*Galanthus nivalis* agglutinin; GNA) linked to an insect derived peptide (neurohormone, venom toxin, etc.) that must normally be delivered to the blood in order to be active (e.g. via a sting). Whereas neither component of the fusion is toxic when fed alone or as a mixture, the fusion protein shows oral toxicity as a result of the carrier transporting the active peptide across the insect gut and delivering it to the blood, from where it can access the central nervous system. The proteins are produced in bacterial or yeast expression systems and delivered orally as a component of diet. Our primary aim was to investigate if peptides derived from the venom of mollusc-hunting cone snails (*Conus spp.*), that had previously been reported to have mollusc-specific activity (conotoxin), could be exploited for the generation of molluscicidal fusion proteins.

Considerable difficulties were encountered with the expression of functional cone snail-derived peptides. Nevertheless, we reported the first successful production of a conopeptide using a yeast-based system. Contrary to published claims, this conopeptide was found to have insecticidal activity and exhibited no toxicity towards slugs. The limited research that has been conducted with molluscs significantly restricted the number of candidates available for exploitation using fusion protein technology. Results obtained from this project have provided promising evidence to suggest that further investment in a biotechnological approach for the development of new molluscicides is worthwhile. An overview of the approach and results are presented in this report but disclosure is limited to protect Intellectual Property.

2. SUMMARY

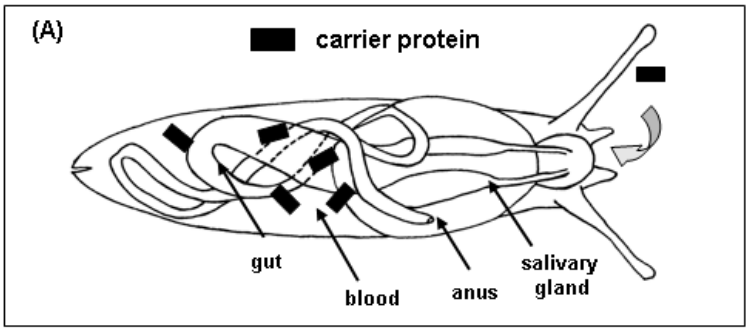
2.1. Introduction

Molluscs, and particularly slugs, are a major problem for agriculture and horticulture in the UK. Reduced crop yields and the cost of applying treatments both have a significant economic impact upon industry. The destruction of seedling embryos and young plants by grey and black field slugs (*Deroceras reticulatum* and *Arion hortensis*, respectively) can result in considerable reductions in overall yield, both directly due to feeding, and also via the generation of gaps in growing crops allowing weeds to grow. Indirect losses due to slug activity can also occur via the vectoring of parasites and plant diseases. The use of molluscicides is widespread, and has increased dramatically over the past 30 years (Brooks & Crook, 2006). Currently only four molluscicides (metaldehyde, methiocarb, aluminium sulphate, ferric phosphate) are approved for the control of snails and slugs in the UK. The grey field slug, *Deroceras reticulatum*, is a major crop pest in the UK, with control by molluscicides requiring widespread treatment. The Pesticide Usage Survey conducted on UK arable crops by FERA, 2008*,* showed, for wheat alone, 882,555 out of a total of 2,068,104 hectares (43%) were treated with molluscicides. The two main active ingredients, metaldehyde and methiocarb (representing 98% of molluscicides used), are toxic to non-target organisms when eaten and are frequent contaminants of ground water. As a result, there is increasing interest in developing more environmentally-friendly alternatives. However, alternatives such as ferric phosphate show lower efficacy and higher cost than metaldehyde-based crop protection methods (Spieser & Kistler 2002). Inclusion of chelating agents such as EDTA in ferric phosphate baits affects earthworm viability (Edwards et al., 2009), and has been reported to cause toxic effects if eaten by mammals (Haldane & Davis 2009). "Natural" molluscicides have also been described, such as garlic (allicin) and copper plate but their effects are limited to temporary repellence (Schoeder et al., 2003). Caffeine has been shown to cause mortality but can also damage the leaves of crops such as lettuce (Hollinsgworth et al., 2002). The nematode species *Phasmarhabditis hermaphrodita,* a known parasite of the grey field slug has been developed as biological control agent with a commercial product now available (Nemaslug®). Field trials with biological control have been conducted on asparagus but the method has shown poor results when compared with chemical molluscicides for mainstream crop protection (Rae et al., 2009).

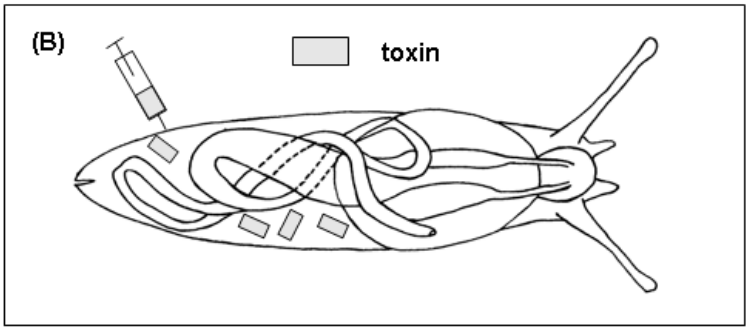
One of the main problems associated with the widespread use of molluscicides is their toxicity towards non-target organisms. The poisoning of wild mammals, domestic animals, agricultural livestock, and birds, has been attributed to metaldehyde and methiocarb slug pellets (Giles et al., 1984, Homeida & Cooke, 1982; Richardson et al., 2003; Greig-Smith et al., 1990; Fletcher et al., 1991: Fletcher et al., 1994). Toxic effects in non-target species can occur both indirectly as a result of feeding upon slugs that have consumed molluscicidal pellets, and directly as a result of feeding on the pellets themselves.

Public concerns about negative environmental impacts resulting from conventional molluscicide usage are reflected in current Government policy objectives. EU directives aim to minimise the use of plant protection products to provide a more environmentally sensitive approach to pest control, whilst retaining the sustainable modernisation of UK agricultural practices. As such, industry has been under increasing pressure to develop alternative approaches for the control of slugs, and during the past twenty years non-chemical control methods have been tested in both agricultural and horticultural sectors (i.e. biological and varietal control methods). However, to date, the uptake of such methods has been hindered by variability in efficacy and high costs, as compared to the use of chemical pesticides (Glen and Moens, 2002). For example, an environmentally benign alternative, biological control of slugs using parasitic nematodes (*Phasmarhabditis hermaphrodita*) has proved expensive and impractical on a large scale. Thus, there is a clear and immediate need for the development of novel, effective and environmentally safe molluscicides as alternatives to conventional chemical control methods for use in both agricultural and horticultural sectors.

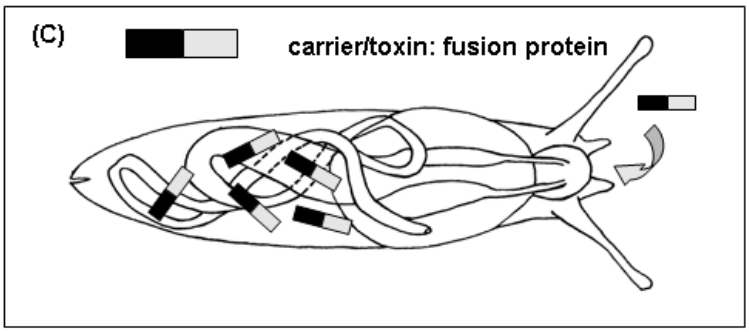
This project was based on the exploitation of a novel approach that allows biologically active proteins, which have low, or no, toxicity when delivered orally to insects to be converted into effective and orally active insecticides. This patented technology has been developed through joint research carried out at the Food and Environment Research Agency (Fera) and Durham University. Recombinant techniques are used to link a gene encoding an insect-specific toxic peptide or protein to a gene encoding a carrier plant protein which is able to cross the gut wall and pass into the circulatory system The recombinant gene encodes a fusion protein containing both components fused into a single polypeptide. The fusion protein is produced using a recombinant expression system, purified, and subsequently ingested by insects after spraying on plant material or as a component of diet. Whereas neither component of the fusion is toxic when fed alone, or as a mixture, the fusion protein shows oral toxicity as a result of the carrier transporting the active peptide across the insect gut and delivering it to the haemolymph, from where it can access its target site. Summary Figure 1 illustrates the fusion protein concept.



(A) Carrier protein when consumed by slugs binds to the gut and is transported across the gut wall and into the blood.



(B) The aim is to produce a protein (using bacterial or yeast cells) that is toxic specifically to molluscs. This toxin will only be effective when injected since it is active only in the blood.



(C) The carrier protein is linked to the toxic protein and a fusion protein is produced (using bacterial or yeast cells). When the fusion protein is fed to slugs, the toxin can be delivered to it's site of action. These fusion proteins are to be incorporated into slug baits.

Summary Figure 1. Diagrammatic representation to illustrate the proposed use of fusion protein technology for the development of novel molluscicidal products.

The aim of this LINK project was to investigate the potential for the extension of this technology for the control of molluscs through the production of novel molluscicidal fusion proteins for the benefit of UK and European agriculture. More specifically our aim was to investigate the potential for the exploitation of naturally occurring molluscicidal proteins or peptides, using methodology based on proven patented fusion protein technology. To this end, a consortium consisting of government and university research laboratories, representing the co-inventors of the fusion protein technology (Fera and Durham University), and industrial partners with experience in protein production (Isagro Ricerca) and in the production and testing of molluscicides (industrial partner 2) was assembled. The central goal of the proposed project was the discovery and development of new target specific

products to be formulated as components of baits for the control of slugs. This project provided an opportunity to develop a novel technology that may lead to an improvement in crop yields for farmers and a reduction in the negative environmental impact of currently approved molluscicides.

2.1.1. Aim and objectives

The overall aim of this project was to conduct collaborative research specifically aimed at the development of novel molluscicidal products based upon fusion protein technology. The scientific objectives of the project were as follows:

1. To evaluate moluscicidal activity and specificity of selected conotoxins for use in fusion proteins, by production as recombinant proteins.
2. To identify other potential mollusc-specific toxins from endogenous and exogenous sources, for use in molluscicidal fusion proteins.
3. To design, produce and assay candidate fusion proteins for efficacy against the grey field slug.
4. To carry out pilot-scale production of a selected fusion protein.
5. To develop optimal bait formulations for the application of pellets in the field.

2.2. Materials and methods

An overview of the materials and methods are presented in this summary report but disclosure is limited to protect Intellectual Property. A wide range of materials and methods were used during this project. These included molecular (DNA cloning and sequencing); biochemical (SDS-PAGE; western blotting; protein purification; high performance liquid chromatography [HPLC]; fast protein liquid chromatography [FPLC]; mass spectrometry MALDI-TOF analysis); fermentation methodology (lab-scale and pilot scale [200 litre]) and physiological studies (laboratory scale bioassays; dissection and extraction).

2.3. Results

2.3.1. Objective I: Production and testing of selected conotoxins for use in fusion proteins

Two conotoxins were selected from the literature based on their reported molluscicidal activity. Initial attempts to express functional peptides using bacteria as an expression host were unsuccessful. Analysis of the purified proteins demonstrated that this was attributable to in-correct folding, known to be critical for biological activity. One of the selected conotoxins was successfully produced using the yeast *Pichia pastoris* as an expression system. This work was published in 2011 in the peer reviewed journal Toxicon and represents the first report of the successful production of a recombinant conotoxin, using yeast as an expression host. Unfortunately, the

purified conotoxin did not show biological activity against *D. reticulatum* but did show activity against two insect species (lepidopteran larvae and dipteran adults).

2.3.2. Objective II: Identification and testing of other mollusc-specific toxins

Three peptides were selected from published literature based on reported specificity of activity towards molluscs. These peptides were reported to disrupt muscle contractions or disrupt the central nervous system of mollusc species. The three peptides were synthesised and tested by injection for activity towards *D. reticulatum*. Disappointingly none of the peptides exhibited any significant effects upon slugs even when injected at high doses. Unfortunately it was not possible to determine if the synthesised peptides contained correctly bridged disulphide bonds and thus the lack of activity observed in injection bioassays may have been attributable to incorrect folding of the peptides.

2.3.3. Objective III: Design and production of fusion proteins containing plant protein carriers and molluscicidal toxins

Two additional carrier proteins, were identified as potential candidates for incorporation into fusion proteins. These proteins were produced in yeast by bench-top fermentation and the purified products were subsequently detected in the circulatory system of slugs in following ingestion. This provided evidence that both proteins were transported across the gut epithelium of slugs demonstrating their potential use as alternative carrier proteins.

2.3.4. Objective IV: Large-scale production of selected molluscicidal fusion proteins

Production scale-up of a candidate protein that showed biological activity towards *D. reticulatum* in laboratory assays was carried out by Actygea, as contracted by Isagro Ricerca. Actygea carried out fermentation at a 200 litre pilot scale. Fermentation was successful, achieving production levels (40-50 mg/litre culture) that were comparable to laboratory scale fermentation that had been conducted by the academic partners. The protein was purified from culture broths using methods that were based on protocols developed by the academic partners. In excess of 3 grams of purified product was purified and verified for biological activity. Actygea also developed a method to allow quantification of product in culture broths. The product was supplied to industrial partner 2 for efficacy testing.

2.3.5. Objective V: Development of optimal bait formulations

Due to the difficulties encountered in the production of large quantities of product, industrial partner 2 carried out only a limited number of trials. In addition to the protein produced by Actygea, the

academic partners produced 500 mg of product for efficacy tests. In a preliminary ‘forced oral route’ oral assay where slugs were force fed an alginate based material containing the product, significant mortality of slugs was recorded. However this result was not reproducible. A second trial did not result in slug mortality although the protein treatment did cause a cessation of feeding by the treated slugs indicating that at least some product was present in active form in the gel. It is thought that storage of the protein containing alginate at low temperature for 3 weeks may have reduced the levels of biologically active product present in the alginate. No molluscicidal activity was observed in subsequent ‘forced oral route’ and bait trials that incorporated protein produced by Actygea into slug baits. Unfortunately the amount of product available for testing was limited and the bait trials conducted by industrial partner 2 were not designed to evaluate effects of the protein containing baits on slug growth or consumption of diet. Thus a comparison with results obtained in laboratory scale assays carried out by the academic partners was not possible. Further work is required to establish if the lack of activity observed in trials using protein containing baits was due to inactivation of the biological activity of product by the pellet formulation process.

2.4. Discussion

The fusion protein approach was originally conceived and developed for the production of novel biopesticidal products for use against insect crop pests. This approach has shown great promise and continues to undergo development through a Technology Strategy Board (TSB) project that aims to develop a pre-registration package for a prototype insecticidal fusion protein. This work represents a truly novel biotechnological approach for the development of biopesticides and the potential for the development of a range of fusion protein products incorporating different toxin and carrier components has been identified. This project aimed to extend the application of this platform technology for the control of slugs.

One major factor limiting the exploitation of this technology for the control of mollusc pests is the limited information published in the field of slug biology. This significantly reduced the number of candidates available for testing. However, promising results have been obtained but are not published here to protect Intellectual Property.

It is concluded that an extensive screening programme covering a wide range of toxins would be required to enable the identification of a mollusc specific molecule. Current commercially available molluscicides are not specific in their action and there are concerns over toxic effects of products not just upon insects but also upon bird and mammals. Candidate toxin and carrier components of fusion proteins are selected based on non-toxicity towards mammals, and as such the development of a fusion protein based product would offer distinct benefits over currently available molluscicides.

A candidate protein was selected for scale-up production and efficacy testing by the industrial partners. The ability to produce a large quantity of a selected protein was limited largely by the expression level of the clones available at the time of pilot scale fermentation. Attempts to improve expression levels through clone selection on high antibiotic containing media and optimisation of fermentation parameters were largely unsuccessful. However, research conducted in a complementary project has demonstrated that expression levels can be significantly enhanced (> 10 fold) using molecular techniques to introduce multiple copies of the expression cassette into the yeast genome. High levels of expression and a simple downstream purification process are both necessary pre-requisites for the production of economically viable biopesticides. A current TSB project is focussed on achieving high levels of fusion protein production and a simple downstream process to enable evaluation of the cost of production of fusion protein products. Nevertheless, the industrial partners, Isagro Ricerca were able to produce in excess of 3 grams of fusion protein and validated the biological activity of the product. Subsequently industrial partner 2 carried out trials against the grey field slug using protein produced both by the academic and industrial partners. The academic partners were able to demonstrate that the protein was still biologically active after incorporation into baits, although determination of the concentration of active protein in the baits was not possible. Results obtained from preliminary 'forced oral route' trials using product supplied by the academic partners were promising and provided evidence for mortality (trial 1) and reduced feeding (trial 1 and 2) following the ingestion of alginate containing protein. Unfortunately trials with product supplied by Isagro Ricerca did not corroborate the initial findings. It remains unclear as to why the 'forced oral route' assay using Isagro Ricerca produced product did not result in a cessation of feeding by the treated slugs. More in depth analysis of slug feeding and growth from the bait trials would have provided valuable data to enable comparisons with data obtained in the laboratory to be made. This was an extremely challenging component of the project. Despite this the industrial partner was able to successfully produce and purify product by fermentation at a pilot production scale. Unfortunately the subsequent analysis of molluscicidal activity in bait trials was hindered by the methods employed and the low efficacy of what was recognised as a 'proof of concept', rather than prototype product. It is concluded that a more effective prototype candidate must be developed in order to enable efficacy in slug bait trials to be evaluated.

Pressure upon the agrochemical industry to produce environmentally compatible crop protection products combined with the withdrawal of actives available for use by the farmer have fuelled research into the development of new approaches to pest control. This project has demonstrated 'proof of concept' for a biotechnological approach suitable for adoption for the development of novel biopesticides. Further investment however is required to develop a truly efficacious product and to enable production at an economically viable cost.