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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

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ABSTRACT

Recombinant fusion protein technology allows specific insecticidal protein and peptide toxins to display activity in orally-delivered biopesticides. The spider venom peptide δ-amaurobitoxin-PI1a, which targets insect voltage-gated sodium channels, was fused to the "carrier" snowdrop lectin (GNA) to confer oral toxicity.

The toxin itself (PI1a) and an amaurobitoxin/GNA fusion protein (PI1a/GNA) were produced using the yeast *Pichia pastoris* as expression host. Although both proteins caused mortality when injected into cabbage moth (*Mamestra brassicae*) larvae, the PI1a/GNA fusion was approximately 6 times as effective as recombinant PI1a on a molar basis. PI1a alone was not orally active against cabbage moth larvae, but a single 30 μg dose of the PI1a/GNA fusion protein caused 100% larval mortality within 6 days when fed to 3rd instar larvae, and caused significant reductions in survival, growth and feeding in 4th – 6th instar larvae. Transport of fusion protein from gut contents to the haemolymph of cabbage moth larvae, and binding to the nerve chord, was shown by Western blotting. The PI1a/GNA fusion protein also caused mortality when delivered orally to dipteran (*Musca domestica*; housefly) and hemipteran (*Acyrthosiphon pisum*; pea aphid) insects, making it a promising candidate for development as a biopesticide.

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1. Introduction

Synthetic pesticides have been widely used for crop protection against herbivorous insects in intensive agricultural production, and are a necessary input to achieve high yields and consequent food security. However, there are widely held concerns over the indiscriminate use of pesticides in general, and insecticides in particular, including the development of resistance in target pests, detrimental effects in non-pest and beneficial insects, contamination of watercourses and the poisoning of higher animals. Therefore, many of the older, broad-spectrum insecticidal compounds have been, or are likely to be, withdrawn (Denholm and Rowland, 1992; Casida and Quistad, 1998; Desneux et al., 2007). Protein-based biopesticides fulfil many of the criteria required for more environmentally compatible approaches to pest control, since they combine efficacy with specificity, and are biodegradable in the environment. Besides naturally occurring protein biopesticides such as *Bacillus thuringiensis* toxins, biotechnological methods can be used to produce recombinant proteins with insecticidal activity. These include insecticidal fusion proteins containing a toxic peptide or protein fused to a "carrier", where the carrier confers oral activity on a toxin that must normally be injected into the insect to reach its site of action, by directing transport of the fusion across the insect gut (Fitches et al., 2004).

Venoms isolated from a range of arachnids have been shown to contain proteins which are biologically active toxins when injected into potential prey. Most are small proteins, in the range 30–70 amino acid residues (variously referred to as peptides or proteins), that principally target neuronal ion channels, and to a lesser extent neuronal receptors and presynaptic membrane proteins, to cause paralysis of the prey (Rash and Hodgson, 2002). As a result of evolutionary selection, some toxins combine a high toxicity for insects with no effects on members of other taxons (Vassilevski et al., 2009). The potency and selective mode of action of spider neurotoxins would make them ideal candidates for use in environmentally compatible pest management technologies, if a suitable delivery system could be devised (Whetstone and Hammock, 2007). In general, these toxins are not effective as oral or contact insecticides, and no system which requires injection could possibly be feasible in the field.

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Biopesticides used for crop protection against insect pests generally function via oral delivery, with the toxin proteins present in, or sprayed on plant tissues susceptible to damage. The use of a "carrier" in recombinant fusion proteins leads to transport of toxin proteins from the gut contents across the insect gut epithelium to the central nervous system where the toxin is active, resulting in dramatically enhanced oral insecticidal activity (Fitches et al., 2002). The mannose-specific lectin from snowdrop (*Galanthus nivalis* agglutinin: GNA) has proved successful as a carrier. It is resistant to proteolytic activity in the insect gut, and can bind to gut epithelial glycoproteins, leading to transport into the haemolymph following ingestion. For example, a toxin protein from the spider *Segestria florentina* was delivered to the haemolymph of lepidopteran larvae after oral delivery by fusing to GNA, causing decreased survival and growth in insects fed on diet containing the fusion protein (Fitches et al., 2004). Fusion proteins are required to possess good stability, so they cannot be degraded in the environment or digested by gut enzymes of pests, and high toxicity, with activity towards pests comparable to the toxin proteins themselves.

δ-Amaurobitoxins, or δ-palutoxins, from the spider *Pireneitega luctuosus* (Araneae: Amaurobiidae; previously referred to as *Paracoelotes luctuosus*) are a family of four similar 36–37 residue peptides, designated Pl1a-d (Corzo et al., 2000). They contain 8 cysteine residues, which are disulphide-linked to form a cysteine knot motif. The δ-amaurobitoxins are effective insecticides, with an estimated LD50 values of 0.95–4.48 μg/100 mg when injected into lepidopteran larvae (oriental leafworm moth; *Spodoptera litura*). They show insect-specific toxicity, with no effects observed for Pl1a, Pl1c and Pl1d after intravenal injection in mice. The amaurobitoxins specifically target insect sodium channels, and their solution structure has elucidated the nature of the interaction (Corzo et al., 2005; Ferrat et al., 2005). The toxin Pl1a was selected as the subject of this study as it combines the highest insecticidal activity with no observed toxicity towards higher animals, and thus would be suitable as a biopesticide.

The present paper reports the production, purification and biological activity of recombinant δ-amaurobitoxin PI1a, and the fusion protein PI1a/GNA comprised of PI1a linked to the N-terminus of GNA. It shows that fusion to GNA enhances the insecticidal activity of Pl1a. Not only was fusion protein more toxic than recombinant PI1a when injected into cabbage moth (*Mamestra brassicae*) larvae, but it also had effective oral toxicity when the toxin alone did not. The fusion protein was also orally toxic to insects of different orders, pea aphids (*Acyrthosiphon pisum*; Hemiptera) and housefly (*Musca domestica*; Diptera). The PI1a/GNA fusion protein has potential to be a useful biopesticide for crop protection in the future.

2. Materials and methods

2.1. Materials

Chemicals and reagents were of analytical grade and were supplied by Sigma or BDH Chemical Company otherwise unless stated. Restriction enzymes and other molecular biology reagents were supplied by Fermentas. A double stranded DNA incorporating a sequence encoding the mature PI1a toxin (P83256), with codon usage optimised for yeast, was designed by the authors, synthesised and supplied by ShineGene Molecular Biotech, Inc. (Shanghai 201109, China; http://www.synthesisgene.com/) in the vector pUC57. Other oligonucleotides were supplied by Sigma Chemical Co. Recombinant snowdrop lectin was produced by the authors by expression in *Pichia pastoris*, as described by Baumgartner et al. (2004).

Mamestra brassicae (cabbage moth) were maintained in a licenced growth facility. The cultures were subject to a 16 h light, 8 h dark cycle and maintained at 25 °C, 40% relative humidity on a standard lepidopteran diet as described before (Bown et al., 1997). *M. domestica* (housefly) larvae were maintained on a wheat flour diet under similar conditions; adults were given 10% sucrose solution *ad libitum*. *A. pisum* (pea aphid) was cultured on plants of *Vicia faba* (broad bean cv. Sutton Dwarf) under conditions of 12 h light, 12 h dark, 18 °C, 70% relative humidity.

2.2. Expression constructs

The Pl1a coding sequence was transferred from pUC57 to the yeast expression vector pGAPZαB (Invitrogen; www.invitrogen.com) by digestion with *Pst*I and *Xba*I, isolation of the coding sequence fragment by agarose gel electrophoresis, followed by ligation to pGAPZαB which had been restricted with the same enzymes. DNA fragments were separated by agarose gel electrophoresis prior to ligation, and were purified from excised gel slices using a QuiaQuick Gel Extraction Kit (Qiagen; www.qiagen.com) as described in the manufacturer's protocol. After ligation, the resulting recombinant plasmid was cloned using standard protocols by transformation of electro-competent cells of *E. coli* (Sambrook and Russell, 2001). Selected clones were checked for correct assembly of the construct by DNA sequencing.

To produce a construct encoding the Pl1a/GNA fusion protein, the mature PI1a coding sequence from a verified expression construct in pGAPZαB was excised by digestion with *Pst*I and *Not*I, and purified by agarose gel electrophoresis as described above. A pGAPZαB plasmid containing the fusion protein construct Hv1a/GNA (Fitches et al., 2012) was digested with *Pst*I and *Not*I to remove the Hv1a coding sequence, and purified by agarose gel electrophoresis. The Hv1a coding sequence was then replaced by Pl1a by ligating the purified fragments, and cloning the resulting recombinant plasmid. Selected clones containing the expression vector encoding the PI1a/GNA fusion protein were verified by DNA sequencing. All DNA sequencing was carried out using Applied Biosystems ABI Prism 3730 automated DNA sequencers by DBS Genomics, School of Biological and Biomedical Sciences, Durham University, UK.

2.3. Expression of PI1a and PI1A/GNA fusion proteins in yeast

pGAPZα plasmids containing the PI1a and PI1a/GNA expression constructs were amplified in *E. coli*, purified and linearized with *Bln*I. Linearised plasmids were transformed into *Pichia pastoris* strain SMD1168H (Invitrogen) using the EasyComp Transformation kit (Invitrogen) as described in the manufacturer's protocol. Transformed yeast clones were plated and selected on YPG agar plates (1% yeast extract (w/v), 2% peptone (w/v), 4% glycerol (v/v), 1.5% agar (w/v)) containing zeocin (100 μg/ml). Selected clones (at least 10 for each construct) were checked for expression of recombinant proteins by analysis of culture supernatant from small-scale shake flask cultures grown for 2–3 days in YPG–zeocin media at 30 °C. Samples of supernatant were separated by SDS-polyacrylamide gel electrophoresis; gels were blotted onto nitrocellulose and probed with anti-$(His)_6$ primary antibodies (Bio-Rad) or anti-GNA primary antibodies, followed by washing, probing with HRP-conjugated secondary antibodies (Bio-Rad), and detection of bound antibodies by ECL, as described previously (Fitches and Gatehouse, 1998).

Selected clones of *P. pastoris* containing the integrated PI1a and PI1a/GNA constructs were grown in a 7.5 L BioFlo 110 bench-top fermenter (New Brunswick Scientific). For fermentation, two 100 ml YPG cultures of *P. pastoris* containing toxin or fusion genes

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