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Effects of *Manduca sexta* allatostatin and an analog on the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphididae) and degradation by enzymes from the aphid gut

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ABSTRACT

The C-type allatostatin, *Manduca sexta* allatostatin (Manse-AS) and the analog $\delta R^3\delta R^5$Manse-AS, where R residues were replaced by their D-isomers, were tested for oral toxicity against the pea aphid *Acyrthosiphon pisum* (Harris) by incorporation into an artificial diet. Both peptides had significant dose-dependent feeding suppression effects, resulting in mortality, reduced growth and fecundity compared with control insects. The $\delta R^3\delta R^5$Manse-AS analog had an estimated LC_{50} of 0.18 μg/μl diet, and was more potent than Manse-AS. At a dose of 0.35 μg $\delta R^3\delta R^5$Manse-AS/μl diet, 98% of aphids were dead within 3 days, at a rate similar to those aphids that had been starved (no diet controls). On comparison, it required 13 days and three times the dose of Manse-AS fed to aphids to attain 96% mortality. It is possible that the feeding suppression effects of Manse-AS on aphids are due to the inhibition of gut motility. The estimated half-life of Manse-AS when incubated with a gut extract from *A. pisum* was 54 min. Degradation was most likely due to cathepsin L cysteine and/or trypsin-like proteases, by an unidentified glutamine-specific protease and by a carboxypeptidase-like enzyme. The D-isomers of R in the Manse-AS analog appeared to prevent hydrolysis by cathepsin L cysteine and trypsin-like enzymes, and enhance its half-life (145 min). However $\delta R^3\delta R^5$Manse-AS was cleaved by enzymes with carboxypeptidase-like and chymotrypsin-like activity. The increased stability of the Manse-AS analog may explain its enhanced feeding suppression effects when continually fed to aphids, and demonstrates the potential use of Manse-AS in a strategy to control aphid pests.

1. Introduction

Aphids have devastating effects on crops as a result of their feeding activities; sucking plant saps, transmitting plant viruses and excreting honeydew, which induces moulds [11]. Aphid populations are usually controlled with chemical insecticides; however this use can result in detrimental consequences for the environment [12], and aphid species are becoming resistant to chemical attack [20,27,31]. Hence there is a need to reduce the usage of these insecticides by introducing safer alternatives with novel modes of action.

The disruption of key physiological processes by insect neuropeptides has been considered as a viable basis for the development of alternative strategies for pest control for many years [25,28,29], but progress has been hindered by problems associated with delivery and stability of these compounds. However, recent developments demonstrating the uptake of neuropeptides across the insect gut and in analog design to enhance their effectiveness suggests neuropeptides may well have a role in a pest management strategy [34–36,44].

One such peptide is the C-type allatostatin Manse-AS (pEVRFRQCYFNPISCF-OH) originally characterized from the lepidopteran *Manduca sexta* L. [30]. This peptide has been identified in a variety of other lepidopterans [6], and orthologues have been deduced from genomic sequence data from the flies *Drosophila melanogaster* (Meigen), *Aedes aegyptii* and *Anopheles gambiae* (Giles), and the beetle *Tribolium castaneum* (Herbst) [42,49,50]. *Manduca sexta*-AS inhibits foregut peristalsis in a variety of lepidopteran larvae [4,6], and when injected into larvae of *Lacanobia oleracea* L. and/or *Spodoptera littoralis* (Hübner), suppresses feeding activity resulting in increased mortality. Although the modes of action of Manse-AS are unknown, it was suggested that mortality was a result of the inhibitory actions of Manse-AS on the gut [2,32]. However, when orally ingested by larvae of *L. oleracea*, Manse-AS is rapidly hydrolyzed by soluble and/or membrane-bound peptides in the gut, resulting in its inactivation [32]. The specific amino acids of Manse-AS susceptible to

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degradation by gut and hemolymph enzymes of *L. oleracea* have been identified so that analogs can be designed to enhance its effectiveness [3,4].

There is little information available regarding the identity of regulatory peptide hormones in the neuroendocrine system of aphids. Tilley et al. [46] detected immunoreactivity to A-type allatostatins and adipokinetic hormone throughout the central nervous system of the vetch aphid *Megoura viciae* Buckton, and suggested that they may serve as central neurotransmitters/modulators in the aphid. Using *in silico* analyses, the sequences of a variety of neuropeptides were reported for the pea aphid *Acyrthosiphon pisum* (Harris), including Proctolin, Kinins, FMRFamides, myoinhibitory peptides, and crustacean cardioacceleratory peptide, and a C-type allatostatin (Table 1), which are all involved in modulating muscle activity [15,47].

Given the roles of neuropeptides in the regulation of foregut motility, there is an interest in their use to suppress feeding of insect pests. This paper reports on the effects of the oral delivery of the C-type allatostatin Manse-AS and the analog $\delta R^3\delta R^5$Manse-AS on the pea aphid, *A. pisum*, as well as the stability of these peptides to gut enzymes.

2. Materials and method

2.1. Aphids

The pea aphid, *A. pisum*, was reared on broad bean (*Vicia faba* L. cv The Sutton), at 20 °C, 50% RH, under a 16 h:8 h light:dark regime.

2.2. Peptides

Manduca sexta allatostatin (pE-V-R-F-R-Q-C-Y-F-N-P-I-S-C-F-OH) and the analog $\delta R^3\delta R^5$Manse-AS (pE-V- δR -F- δR -Q-C-Y-F-N-P-I-S-C-F-OH), in which the L-isomer of the arginine residues (R) at positions 3 and 5 were replaced by the D-isomer (δR), were custom synthesized at the Advanced Biotechnology Centre, Imperial College, London, UK. Both peptides have a di-sulphide bridge linking C^7 and C^{14}. All other chemicals were obtained from either Sigma–Aldrich (UK) or BDH (Poole, UK). Peptides were quantified using reversed-phase high performance liquid chromatography (RP-HPLC).

2.3. Aphid artificial diet assays

An artificial diet suitable for the development and parthenogenetic reproduction of *A. pisum* was prepared according to Febvay et al. [24]. Aphid bioassays were set up using ten neonate (0–24 h old) *A. pisum* nymphs per feeding chamber (replicate). The nymphs were produced parthenogenetically by apterous females feeding on artificial diet overnight. Feeding chambers were set up as previously described [21,23,38]. Briefly, the feeding chambers consisted of the base of a 35 mm Petri dish lined with moist filter paper. Two layers of Parafilm M® were stretched over the top, with 100 μl of artificial diet sandwiched between the two layers. Unless otherwise stated, a total of 50 aphids (5 replicates (n), 10 insects per replicate) were used for each treatment for the bioassays. Feeding chambers were kept at 20 °C, 50% RH, under a 16 h:8 h light:dark regime, and fresh diet sachets (sandwich layers) were provided every 3 days to avoid fungal and bacterial contamination.

Manse-AS was dissolved in 80% acetonitrile/0.01% TFA and added to the diet at final concentrations of 0.1 μg/μl and 1 μg/μl. The $\delta R^3\delta R^5$Manse-AS was also dissolved in 80% acetonitrile/0.01% TFA and added to the diet at final concentrations of 0.035 μg/μl and 0.35 μg/μl. Control treatments included diet only and a 'no diet' control (no diet or water provided) to ascertain how long *A. pisum* survived without food. Control diets had comparable amounts of 80% acetonitrile/0.01% TFA added. The acetonitrile/0.01% TFA was then evaporated from all the diets by centrifugal evaporation and the diet made up to the original volume using distilled water. As a further control, the combined individual amino acid constituents of the Manse-AS peptide at the equivalent concentration of 1 μg/μl of Manse-AS were added to diet and tested, using standard diet as the control.

The number of aphids feeding per replicate on the diets was recorded every hour over the initial 6 h of the bioassay. After this point the numbers feeding per replicate were recorded at one daily timepoint. The number of aphids alive per replicate and the number of nymphs produced once the aphids reached maturity were recorded daily. The bioassays were terminated after 13–16 days. The surviving aphids were photographed at regular intervals during development (typically on days 3, 6 and 9) using a Nikon Coolpix 4500 digital camera, attached to a binocular dissecting microscope. The length (from head to cauda) and width (across the metathorax) of the aphids were measured using Image J computer software. A 10 mm scale was photographed under the same magnification, using the same camera settings, and this was used to calibrate the measurements.

Lethal concentration (LC) values were calculated by probit analysis for the mortality of *A. pisum* after ingesting diet containing $\delta R^3\delta R^5$Manse-AS for 3 days. Fifty aphids were used for each dose tested, and feeding chambers containing neonate nymphs were set up as described above. Five doses of $\delta R^3\delta R^5$Manse-AS were tested ranging from 0.035–0.35 μg/μl. As the peptide had a tendency to precipitate out of solution once it was added to the artificial diet, the final concentrations of peptide in the diet were quantified as described in Section 2.7.

2.4. Preparation of gut extract

The entire digestive tracts were dissected from adult *A. pisum*, rinsed in saline (0.15 M NaCl) and immediately placed into an Eppendorf tube containing ice-cold saline at a ratio of one digestive tract per 4 μl saline. Tissues were sonicated (MSE soniprep 150 ultrasonic disintegrator (Jencons (Scientific) Ltd., Forest Row, UK) and centrifuged at 10,000 × g at 4 °C for 15 min. The resulting supernatant (=gut extract) was removed, aliquoted into fresh Eppendorf tubes and stored at −85 °C.

2.5. Enzyme activity of gut extract

Gut extract was tested for aminopeptidase and cathepsin L cysteine protease activity [16] prior to peptide degradation studies to optimize conditions. Aminopeptidase activity was assayed using 0.5 mM LpNA (L-leucine-*p*-nitroanilide; Sigma–Aldrich. UK) as a substrate and cysteine protease activity was assayed using 0.5 mM Z-Phe-Arg-pNA (benzoyloxycarbonyl-L-phenylalanyl-L-arginine-*p*-nitroanilide; Bachem (UK) Ltd.) as a substrate [16,22] in McIlvaines citrate-phosphate buffer containing 3 mM EDTA and 3 mM cysteine, pH 6.0. This pH is comparable to the pH range (6.0–8.0) of the gut contents of *A. pisum* [16]. The change in absorbance at 405 nm was recorded at 20 °C in a microtitre plate reader (Bio-

Table 1
A comparison between the amino acid sequence of *M. sexta* allatostatin (Manse-AS) and the predicted sequence of *A. pisum*—C-type allatostain.

Species	Sequence															
Manduca sexta	pE	V	R	F	R	Q	C	Y	F	N	P	I	S	**C**	F	OH
Acyrthosiphon pisum		S	Y	W	K	Q	C	A	F	N	A	V	S	C	F	Amide

The darker shading identifies identical amino acids, the lighter shading conservative substitutions.