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Transepithelial flux of an allatostatin and analogs across the anterior midgut of *Manduca sexta* larvae *in vitro*

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ABSTRACT

The transepithelial flux of cydiastatin 4 and analogs across flat sheet preparations of the anterior midgut of larvae of the tobacco hawkmoth moth, *Manduca sexta*, was investigated using a combination of reversed-phase high-performance liquid chromatography (RP-HPLC), enzyme-linked immunosorbent assay (ELISA) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The lumen to hemolymph (L–H) flux of cydiastatin 4 was dose and time-dependent, with a maximum rate of flux of c. 178 pmol/cm^2/h) measured after a 60-min incubation with 100 μmol/l of peptide in the lumen bathing fluid. The rates of flux, L–H and H–L, across the isolated gut preparations were not significantly different. These data suggest that uptake across the anterior midgut of larval *M. sexta* is via a paracellular route. Cydiastatin 4 was modified to incorporate a hexanoic acid (Hex) moiety at the N-terminus, the N-terminus extended with 5 P residues and/or the substitution of G^7 with Fmoc-1-amino-cyclopropylcarboxylic acid (Acpc). The incorporation of hexanoic acid enhanced the uptake of these amphiphilic analogs compared to the native peptide. Analogs were also more resistant to enzymes in hemolymph and gut preparations from larval *M. sexta*. A modified N-terminus gave protection against amino-peptidase-like activity and incorporation of Acpc inhibited endopeptidase-like activity. Although analogs were stable in the hemolymph, they were susceptible to amidase-like activity in the gut, which appears to convert the C-terminal amide group to a free carboxylic acid, identified by an increase in 1 mass unit of the peptide analog.

1. Introduction

The use of orally administered insect neuropeptides for a biorational approach to pest control is considered not to be effective due to their poor absorption characteristics in the alimentary canal and their rapid degradation by gut enzymes. However, a number of studies have demonstrated that peptides and proteins are able to penetrate the gut epithelium of an insect in an active form. The peptides proctolin, trypsin-modulating factors (TMOF) and pheromone biosynthesis-activating peptide (PBAN) have all been detected in the insect hemolymph and found to be biologically active after oral administration [11–13,33,38]. Proteins, such as albumin and the lectin *Galanthus nivalis* agglutinin (GNA) have also been shown to traverse the insect gut [14,20,31], and the latter has been used as a recombinant fusion protein, comprised of *Manduca sexta* allatostatin attached to GNA, to deliver allatostatin across the insect gut following oral ingestion [21]. Modifications to the primary structure of peptides, using amino acid mimics and fatty acid moieties, have also been undertaken to enhance their stability to enzymes in the gut, and their ability to penetrate the gut epithelium [27–30,34].

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Oral delivery of peptides, or biologically active analogs, to the insect hemolymph could result in an endocrine imbalance and disruption of the physiological processes that peptides regulate as a means of insect pest control, such as feeding, growth and development. Cydiastatin 4, an A-type allatostatin, which has been identified in various Lepidoptera including *M. sexta* [6–8,16,17,19], has been shown to inhibit foregut peristalsis in larvae of the tomato moth, *Lacanobia oleracea in vitro* [8,18,19,24]. This peptide is localized in the frontal ganglion and nerves of the stomatogastric nervous system that innervate the muscles of the gut, suggesting a role in the regulation of feeding activity [19]. Disruption of feeding activity could be a means to control lepidopteran pests; injection of the C-type allatostatin, *M. sexta* allatostatin (Manse-AS), into *L. oleracea* larvae suppresses feeding activity resulting in death [3]. However, before such a strategy can be considered for practical use by oral application, it is first necessary to understand the stability and uptake of an allatostatin in the gut of a larval lepidopteran so that suitable analogs can be designed to enhance the effects of the native peptide.

The focus of this study was to investigate, using *in vitro* midgut preparations from *M. sexta* larvae, the transepithelial flux of cydiastatin 4 and analogs designed to enhance the stability and ability of this peptide to penetrate the gut epithelium.

2. Materials and methods

2.1. Experimental animals

M. sexta were reared from eggs supplied by Prof. S. Reynolds, University of Bath, using methods described by Yamamoto [36]. Day 2 fifth stadium larvae were used in all experiments, and were anesthetized by submersion in ice-cold water before use. *L. oleracea* was reared as described by Audsley et al. [3]. Sixth stadium larvae were used for gut bioassays.

2.2. Physiological saline

Saline used was based on that described for *M. sexta* by Chamberlin [15] and contained in mmol/l: 5 $MgCl_2$; 1 $CaCl_2$; 5.8 KOH; 6 Na_2HPO_4; 7.7 K^+-citrate; 2.8 Na^+-succinate; 10 glucose; 180 sucrose; 3.6 alanine; 9.4 glutamine; 12.8 glycine; 9.7 histidine; 5.6 malic acid; 7.4 proline; 8.9 serine; 4.6 threonine; 10 MOPS. The pH of the saline was adjusted to 6.7 with nitric acid.

For foregut peristalsis assays the composition of saline was (mmol/l) 154 NaCl; 2.7 KCl; 1.8 $CaCl_2$; 22 glucose and 12 hydroxyethylpiperazine ethanesulphonic acid. The pH of the saline was adjusted to 7.2 [24].

2.3. Synthetic allatostatin

Cydiastatin 4 (ARPYSFGL-amide) and analog cydiastatin 4α (PPPPPARPYSFGL-amide) were custom synthesized at the Advanced Biotechnology Centre, Imperial College, London, UK. Analogs cydiastatin 4β (PPPPPARPYSF[Acpc]L-amide), cydiastatin 4γ (Hex-ARPYSF[Acpc]L-amide) and cydiastatin 4δ (Hex-PPPPPARPYSF[Acpc]L-amide), incorporating Fmoc-1-amino-cyclopropylcarboxylic acid ([Acpc]) and or hexanoic acid (Hex), were synthesized according to previously described procedures [27].

2.4. Midgut tissues for transepithelial flux studies: flat-sheet preparations

Anterior midguts from larval *M. sexta* were carefully dissected free of fat body and tracheal connections, cut at the junction with the foregut and at the position where the attached Malpighian tubules loop back to the posterior midgut (at the first abdominal segment). Each tissue was opened as a flat sheet and mounted into Ussing-type chambers. The tissue was bathed bilaterally in 1 ml physiological saline at 30 °C, and mixed by bubbling with 100% oxygen. The design of the Ussing-type chambers ensured that there were different saline levels at the two sides of the chamber, which could be used to determine whether there was fluid leakage across the gut epithelium due to tissue damage. After two saline changes, cydiastatin 4 or a cydiastatin 4 analog was added to the lumen side of the tissue. The lumen to hemolymph (L–H) flux was measured by taking aliquots of saline from the hemolymph side of the tissues at different time intervals and analyzing by liquid chromatography and enzyme-linked immunosorbent assay (ELISA). To measure the hemolymph to lumen (H–L) flux of cydiastatin 4, peptide was added to the hemolymph side of the midgut, and aliquots of saline were removed from the lumen side of the tissue for analysis.

At the end of each experiment, 1% amaranth, which is not absorbed across gut epithelia, was added to the lumen side of the flat sheet preparations to check for damage to the tissues that would allow leakage of peptide across the tissue [38].

Analysis of HPLC fractions by MALDI-TOF MS was used to confirm transepithelial flux of intact peptide and/or metabolic products.

2.5. High-performance liquid chromatography

Samples were diluted with 0.1% TFA and loaded, via a Rheodyne loop injector, onto a Jupiter C_{18} 10 μm 300 Å narrow bore column (250 mm × 2.1 mm i.d.; Phenomenex, Macclesfield, UK) fitted with a guard column (30 mm × 2.1 mm i.d.) of similar packing material. The column was eluted with a linear gradient of 10–50% acetonitrile/0.1% TFA, over 40 min at a flow rate of 0.2 ml/min, and elution monitored at 214 nm, using a Beckman 32 Karat chromatographic system (Beckman Coulter (UK) Ltd.), comprising a dual pump programmable solvent module 126 and a UV detector module 166. Fractions (1 min, 0.2 ml) were collected and concentrated to c. 10 μl by centrifugal evaporation for mass analysis. The elution positions of cydiastatin 4 and analogs were determined so that ELISA and mass analysis could be conducted on the appropriate fractions.

2.6. Mass analysis of allatostatin and degradation products

Mass spectra were acquired on a Voyager DE STR MALDI-TOF mass spectrometer (Applied Biosystems, Warrington, UK). The