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Metabolism of cydiastatin 4 and analogues by enzymes associated with the midgut and haemolymph of *Manduca sexta* larvae

Neil Audsley [a,*], June Matthews [a], Ronald Nachman [b], Robert J. Weaver [a]

[a] *Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK*
[b] *Southern Plains Agricultural Research Centre, U.S. Department of Agriculture, 2881 F/B Road, College Station, TX 77845, USA*

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Abstract

The degradation of synthetic cydiastatin 4 (ARPYSFGL-amide) and cydiastatin 4 analogues cydiastatin 4α (PPPPPARPYSFGL-amide) and cydiastatin 4β (PPPPPARPYSF[Acpc]L-amide) by enzymes associated with the midgut and/or haemolymph of the tobacco hawkmoth moth, *Manduca sexta* was investigated using reversed-phase high performance liquid chromatography (RP-HPLC) combined with matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). Cydiastatin 4 had an estimated half-life of *c*. 16.5 min when incubated with midgut tissue *in vitro* and *c*. 2.5 min with midgut lumen contents. Two degradation products were identified; cydiastatin$^{1-6}$, due to cleavage of the C-terminal di-peptide GL-amide, and cydiastatin$^{2-8}$, due to cleavage of the N-terminal A residue. Both cydiastatin 4α and cydiastatin 4β had increased stability to gut and haemolymph enzymes, and full biological activity, but reduced potency compared to cydiastatin 4 when assayed on foregut peristalsis. The P-extended N-terminus of both analogues prevented hydrolysis by aminopeptidases and the replacement of the susceptible G residue with cyclopropylalanine ([Acpc]) counteracted carboxypeptidase activity. However, both analogues were susceptible to amidase-like activity giving an increase in one mass unit presumably due to the conversion of the C-terminal amide group to the free carboxylic acid. No metabolism of cydiastatin 4β occurred when incubated with larval *M. sexta* haemolymph over a 90 min period.

Keywords: Neuropeptide; MALDI-TOF; Mass spectrometry; Insect; Cyclopropylalanine; Allatostatin

1. Introduction

Insect neuropeptides are important regulators of most physiological processes in insects, including salt and water balance, regulation of energy metabolism, contractions of visceral muscles, and growth and development through their effects on ecdysteroid and juvenile hormone biosynthesis (reviewed by Gäde et al., 1997). Recent advances in mass spectrometry and insect genome sequence projects have significantly advanced our knowledge of the structure and distribution of insect neuropeptides and created further opportunities to investigate their endocrine functions. Disruption of the endocrine system and the use of insect neuropeptides for a more biorational approach to pest control have been repeatedly discussed over the last two decades (Keeley and Hayes, 1987; Masler et al., 1993; Hoffmann and Lorenz, 1998; Gäde and Goldsworthy, 2003). However, it is believed that insect neuropeptides are unsuitable candidates for pesticides because they cannot be used directly due to their chemical properties. If applied topically they are unlikely to penetrate the insect cuticle and would be rapidly degraded by gut enzymes if consumed with a food source (Keeley and Hayes, 1987; Nachman et al., 2002a). In addition, orally delivered peptides would have to penetrate the gut tissues to reach the haemolymph for transport to their sites of action. Once in the haemolymph they would also be susceptible to circulating enzymes (Audsley et al., 2002a; Garside et al., 1997a; Peralta et al., 2000). Peptides and proteins, have however, been shown to penetrate the gut epithelium of an insect. Proctolin, trypsin modulating

* Corresponding author. Fax: +44 (0) 1904 462111.
E-mail address: n.audsley@csl.gov.uk (N. Audsley).

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factor (TMOF) and pheromone biosynthesis activating peptide (PBAN) were detected in the haemolymph of various insects after oral administration demonstrating they were able to penetrate the gut tissue intact (Raina et al., 1994; Bavoso et al., 1995; Borovsky and Mahmood 1995; Zhu et al., 2001). Proteins, such as albumin, green fluorescent protein and the lectin *Galanthus nivalis* agglutinin (GNA) have also been shown to traverse the insect gut *in vivo* (Powell et al., 1998; Fitches et al., 2001; Habibi et al., 1992; Casartelli et al., 2005). In addition *Manduca sexta* allatostatin has been delivered to the haemolymph of larval *Lacanobia oleracea* following oral ingestion by being attached to GNA (Fitches et al., 2002). Insect neuropeptide analogues have also been designed that are more stable in the gut and haemolymph, or have enhanced hydrophobicity so they can penetrate the insect cuticle (Nachman et al., 1998, 1999, 2001, 2002a,b). Oral delivery of peptide analogues with enhanced stability and/or hydrophobicity so they can reach their target receptors in an active form can be a means of disruption of the endocrine balance and the physiological processes that are normally regulated by the native peptide hormone. This may lead to the development of innovative strategies to target insect pest species. A thorough understanding of the degradative pathways in the gut and in the haemolymph is required to aid the design of peptide analogues that can resist enzyme attack, as well as retain biological activity.

The A-type allatostatin family of insect peptides have been identified in numerous insect species including various Lepidoptera (Gäde et al. 1997). They are usually 6–18 amino acids in length and are characterised by their conserved C-terminal pentapeptide (Y/FXFGL-amide), which represents the minimum requirement for biological activity. This family of allatostatins inhibits juvenile hormone biosynthesis in cockroaches (Weaver et al., 1998), but in lepidopteran larvae they are myo-inhibitory on the foregut (Audsley et al., 2005; Duve et al., 1997a, b, 1999, 2000). Their localisation in the frontal ganglion and in the muscles supplying the crop and stomodeal valve of larval Lepidoptera suggests these A-type allatostatins have a role in the control of feeding (Audsley et al., 2005; Duve et al., 2000).

The focus of this study was to investigate the hydrolysis of a representative lepidopteran A-type allatostatin, cydiastatin 4 (ARPYSFGL-amide), by tissue bound and soluble enzymes from larval *M. sexta* using liquid chromatography and mass spectrometry. This information was then used to develop biologically active analogues with enhanced enzyme resistance.

2. Materials and methods

2.1. Experimental animals

Manduca sexta were reared from eggs supplied by Prof. S. Reynolds, University of Bath, using methods described by Yamamoto (1969). Day two fifth-instar larvae were used for all enzyme studies, and were anaesthetised by submersion in ice-cold water before use. *Lacanobia oleracea* were reared as described by Corbitt et al. (1996). Sixth stadium larvae were used for gut bioassays.

2.2. Physiological saline

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 12 (Cook and Holman, 1978). The pH of the saline was adjusted to 7.2.

Saline used for peptide metabolic studies was based on that described for *M. sexta* by Chamberlin (1989) 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.

2.3. Synthetic allatostatin

Cydiastatin 4 (ARPYSFGL-amide) and analogue cydiastatin 4α (PPPPPARPYSFGL-amide) were custom synthesised at the Advanced Biotechnology Centre, Imperial College, London, UK. Analogue cydiastatin 4β (PPPPPARPYSF[Acpc]L-amide, incorporating cyclopropylalanine ([Acpc]), was synthesised according to previously described procedures (Nachman et al., 1999).

2.4. Foregut contractions

Sixth instar *L. oleracea* larvae were starved overnight, anaesthetised with CO_2 and cut open along their dorsal surface to one side of the heart from the head to the third abdominal segment. The cuticle was pinned back to expose the foregut and anterior midgut. After rinsing several times with physiological saline, the gut was bathed in 200 μl of fresh saline at 22 ± 2 °C and the preparation viewed under a dissecting microscope to observe peristaltic contractions of the foregut. A baseline frequency of contractions was established over 2×1 min periods. Control saline was then replaced by saline containing either cydiastatin 4 or analogues at various doses. Frequency of contractions was again counted over 2×1 min periods, after which the gut was washed several times to remove peptides and return the foregut to baseline peristalsis. Once this was achieved, another solution of saline + peptide could be added and foregut peristalsis observed again.

2.5. Extract of anterior midgut tissue

Midguts from *M. sexta* larvae were dissected in physiological saline. Guts were opened as a flat sheet, cut posterior of the stomodeal valve, and anterior section of the midgut removed. This was washed several times in saline to remove gut contents and haemolymph, sonicated in ice-cold saline using a MSE soniprep 150 ultrasonic disintegrator (Jencons (Scientific) Ltd., Forest Row, UK), and centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was removed and stored at −70 °C until required. Protein content of gut preparations was determined using a microtitre based Bradford assay (Bio-Rad Laboratories Ltd., Hemel Hemstead, UK). Gut extract was diluted with saline to 2 μg protein/100 μl for assay.

2.6. Preparation of midgut lumen contents

The midguts of five larval *M. sexta* were ligated at the anterior and posterior ends, removed, washed in physiological saline, and then opened to remove the lumen contents. The contents were diluted approximately 10-fold in ice-cold physiological saline, vortexed, centrifuged and stored as described above for midgut tissues. Preparations were diluted to a protein concentration of 1 μg in 100 μl physiological saline for assay.