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Structures, functions and occurrence of insect allatostatic peptides

Introduction

The multitude of physiological processes controlled or modulated by insect juvenile hormones (JH) underlines the vital roles performed by these sesquiterpenoid hormones in insects. Predominant among the various functions of JH is the control of metamorphosis in pre-adult stages, and the regulation of reproductive physiology (especially oocyte growth) in adults. In addition, these hormones have been implicated in the determination of phase and caste polymorphisms, diapause induction, pheromone production, and several aspects of behaviour (for a recent review, see Nijhout, 1994). Furthermore, in many of those circumstances where JH-controlled systems have been investigated, there are marked changes in JH levels that can be correlated with changing physiological events. For example, in cockroaches, changes in the biosynthetic activity of the corpora allata (CA) are directly related to corresponding changes in haemolymph or whole-body JH levels (Tobe *et al.*, 1985; Edwards *et al.*, 1990) and these changes can be correlated with specific physiological events. These and other studies have also pointed to the fact that JH levels may change dramatically over relatively short periods of time. Ultimately, the finite haemolymph or tissue levels of JH is a balance between the rate at which the hormones are biosynthesised by the CA (since JH is released immediately following biosynthesis), and the rate at which these molecules are metabolised. Despite the fact that there is ample evidence that JH-degrading enzymes (predominantly, esterases and epoxide hydrolases) can play a role in determining the physiological levels of JH, it is likely that the overall control of tissue or haemolymph JH levels is more intimately linked with changes in the rate of JH biosynthesis (Tobe & Stay, 1985; Tobe *et al.*, 1985). Thus, the control of JH biosynthesis by the CA is, ultimately, the factor that modulates all JH-mediated physiological events.

The CA are retrocerebral endocrine organs that are innervated by neurones originating in the brain. Although some of these connections may be nervous, there is ample evidence that they are also neurohormonal, and that neurosecretory material passes from the neurosecretory cells of the brain

directly to the CA (Scharrer, 1952; Tobe & Stay, 1985; Cassier, 1990). In many insect species, these nerves pass through the corpora cardiaca, which are themselves often in intimate association with the CA. In some species, the CA receive afferent and efferent connections from the suboesophageal ganglion (SOG). In addition, the CA are bathed in haemolymph and therefore are exposed to regulatory factors circulating in the haemolymph.

The insect brain is in direct receipt of both internal and external stimuli that provide a constant stream of information about the physiological conditions prevailing in the internal and external environment. Many of these stimuli will ultimately result in a physiological response to changes in the environment and, frequently, this response will involve modification of the endogenous JH levels. It is therefore not surprising that investigators seeking the factors controlling CA activity have concentrated their efforts on the association between the brain and retrocerebral complex and, in particular, on the neurohormones produced by the brain neurosecretory regions. Deducing from a considerable body of evidence that surgical section of the nervous connections between brain and CA could alter the rate of JH biosynthesis, and from experiments in which crude brain extracts were shown to alter the activity of the CA, researchers concentrated on the brain as the primary source of allatoregulatory factors. These early studies were greatly aided by the development of the *in vitro* JH biosynthesis assay (Pratt & Tobe, 1974; Tobe & Pratt, 1974) in which changes in the rates of JH biosynthesis are monitored by measuring changes in the rate of production of JH incorporating a radiolabelled precursor (radiolabelled [*methyl*] methionine). From such studies, it soon became apparent that brain tissue contained principles that were able either to stimulate or to inhibit JH production by the CA. These biologically active molecules were termed allatotropins and allatostatins (ASTs), respectively.

The first allatostatins to be characterised were isolated from brains of the cockroach *Diploptera punctata* (Woodhead *et al.*, 1989; Pratt *et al.*, 1991). Several closely related allatostatins have subsequently been isolated from other cockroach species including *Periplaneta americana* and *Blattella germanica* (Bellés *et al.*, 1994; Weaver *et al.*, 1994). In addition, similar peptides have been detected in a dipteran species (the blowfly *Calliphora vomitoria*; Duve *et al.*, 1993). In some species (for example, *D. punctata*), the allostatins are widely distributed in the tissues, and have been isolated or immunochemically localised in the brain, haemolymph, visceral ganglia, gut and other nervous or neuromuscular organs (Woodhead, Stoltzman & Stay, 1992; Stay *et al.*, 1994a; Stay, Tobe & Bendena, 1994b; Tobe, Yu & Bendena, 1994, Yu *et al.*, 1995). These cockroach peptides share a characteristic, highly conserved C-terminus, and show a remarkable conservation of amino acid sequence, even in different cockroach species. From the known sequences of the *D. punctata* allatostatins, researchers designed probes that

eventually led to the isolation and characterisation of the gene coding for allatostatins in *D. punctata* and, subsequently, in *P. americana* (Donly *et al.*, 1993; Ding *et al.*, 1995). These genes encode 13 or 14 related peptides, respectively. All of these peptides have been synthesised and all show some degree of allatostatic activity *in vitro* against CA from *D. punctata* and *P. americana*. However, glands from different stages of *P. americana* do not show the same degree of sensitivity to synthetic *Diploptera* allatostatins irrespective of physiological status, and some of the peptides are potent inhibitors of JH biosynthesis at certain times, whereas others are not (Weaver *et al.*, 1995b). Nevertheless, probes based either on native peptides or on the encoding gene have been utilised in the search for related sequences in other organisms (Stay *et al.*, 1994b; Tobe *et al.*, 1995).

Another peptide with allatostatic activity has been identified in the moth *Manduca sexta* (Kramer *et al.*, 1991). This peptide, containing 15 amino acid residues, bears no sequence similarity to the cockroach allatostatins. Using probes based on the amino acid sequence of *M. sexta* allatostatin (Mas-AST), Tobe *et al.* (1995) and Jansons *et al.* (1996) have reported the existence of a Mas-AST-like gene in the noctuid moth *Pseudaletia unipuncta*. Similar results, using the same probe, have recently been obtained in another noctuid (*Lacanobia oleracea*), in which this probe has been shown to hybridise with mRNA isolated from larval and pupal brains (J. P. Edwards *et al.*, unpublished results). However, it is clear that the cockroach peptides are inactive as allatostatins in *M. sexta*, and likewise, the *M. sexta* allostatin shows no inhibition of JH biosynthesis by CA of the cockroach *P. americana* (Kramer *et al.*, 1991; R. J. Weaver, unpublished results). These results suggest that the Dictyoptera and Lepidoptera each utilise a separate type of allatostatic neuropeptide. However, this interpretation is complicated by the observations that the cockroach allatostatins are present in representatives from other insect taxa, including Diptera, Hymenoptera and Lepidoptera and, indeed, in other non-insect groups such as Crustacea, Arachnida and Mollusca (see Stay *et al.*, 1994b and references contained therein). Similarly, the *M. sexta* AST appears to occur in at least one other insect species – the dipteran *Drosophila melanogaster* (Zitnan, Sehnal & Bryant, 1993; Tobe *et al.*, 1995). In many of these examples, it is unlikely that the primary function of these molecules is allatostatic. Equally, the fact that the cockroach allatostatins are widely distributed in the peripheral nervous system, often in association with muscular tissues, suggests at least one other function for these molecules as modulators of neuromuscular function. Added to these complications is the discovery of yet another allatostatic substance in the moth *M. sexta* which appears to be a much larger peptide than Mas-AST, and which, unlike the cockroach allatostatins, appears to act slowly and in a non-reversible fashion prior to metamorphosis (Bhaskaran *et al.*, 1990; Unni *et al.*, 1993).

Despite the progress made in the identification of allatostatic neuropeptides from several insect species, a host of questions remain to be answered. Why do cockroaches appear to require more than a dozen different peptides (all apparently performing similar functions) whereas the Lepidoptera seem to need only one or two? Why do flies have peptides identical or similar to both cockroach and moth allatostatins, when these peptides seem to be devoid of allatostatic activity in adult dipterans? Because these peptides occur in insects in which they do not appear to be allatostatins, yet do have myomodulatory activity more or less universally, are they indeed the exclusive or only primary modulators of CA activity in cockroaches or moths? This review concentrates exclusively on the allatostatins, although at least one peptide with allatotropic activity has also been identified (Kataoka *et al.*, 1989). Studies that led to the elucidation of the structures of those molecules so far identified from insects are reviewed, and the temporal and physical distribution of these molecules and of the genes that encode the peptides are considered. The question of the role(s) of these peptides is addressed, and their mode of action and metabolism considered. Finally, an attempt is made to address some of the outstanding questions raised in this brief introduction.

Cockroach allatostatins

Early experiments, particularly those with cockroaches, provided clear indications that insect brains exerted an inhibitory effect on JH production and were most likely a major site of production of allatostatic signals acting on the CA (Scharrer, 1952; Engelmann, 1957, 1959; Stay & Tobe, 1977, 1978). Direct evidence for the occurrence of cockroach allatostatins came from *in vitro* assays of aqueous extracts of brain and retrocerebral tissue of virgin female *D. punctata* (Rankin *et al.*, 1986). Using direct radiochemical measurements of JH biosynthesis (Pratt & Tobe, 1974; Tobe & Pratt, 1974), CA were observed to be inhibited in a dose-dependent fashion by extracts of corpora cardiaca and also by 0.5 brain equivalents of a water soluble, heat-stable and trypsin-sensitive substance from protocerebral lobes (Rankin *et al.*, 1986). Subsequent studies showed that CA-inhibitory, neuropeptide-like materials were extractable from several ganglia of the central nervous system of females at different stages during the reproductive cycle (Rankin & Stay, 1987). Aqueous extracts of brains of adult males, and of final instar male and female larvae, were similarly shown to inhibit release of JH from adult female CA (Paulson & Stay, 1987; Paulson *et al.*, 1987).

Following the demonstration that simple extracts of brain tissue were able to inhibit JH biosynthesis in a reliable assay, it became possible to proceed with the isolation of functional allatostatic factors. Initially, four peptides

showing strong and rapid inhibition of JH biosynthesis were isolated and characterised from high-performance liquid chromatography (HPLC) separations of extracts of brains from virgin female *D. punctata* (Woodhead *et al.*, 1989). Each of these peptides, termed allatostatins I–IV, was C-terminally amidated. They show a high degree of C-terminal identity and range in size from 8 to 18 amino acid residues (Table 1). Using similar techniques, three additional *D. punctata* allatostatins have been identified from extracts of brains of adult female *D. punctata* (Pratt *et al.*, 1991; Woodhead *et al.*, 1994) and two more from the cockroach *P. americana* (Weaver *et al.*, 1994). Between 1000 and 4500 cockroach brains were required to purify each batch of allatostatic peptides. Yields of allatostatin per cockroach brain ranged from 0.1 pmol brain^{-1} in *D. punctata* (Woodhead *et al.*, 1989) to 0.3 pmol brain^{-1} in *P. americana* (Weaver *et al.*, 1994). Four additional cockroach allatostatins, one identical to *D. punctata* allatostatin IV, were subsequently isolated and sequenced from brains of virgin adult females of a third species, *Blattella germanica* (Bellés *et al.*, 1994).

Table 1 summarises data on the cockroach allatostatins isolated thus far, their origins and their past and current designations. These peptides clearly belong to an extended intraphyletic family that shares the C-terminal sequence Tyr/Phe-Xaa-Phe-Gly-Leu/Ile-NH_2, where Xaa is alanine, glycine, serine or asparagine. Bioassays of preliminary reverse-phase separations indicated that the five *D. punctata* allatostatins and two *P. americana* allatostatins represented only a portion of the extractable JH-inhibitory activity in adult female cockroach brain of either species. The likelihood that additional allatostatins from the same family could be found in these extracts was confirmed by the molecular characterisation of a multiple allatostatin precursor cDNA and gene in *D. punctata* that encoded 13 very similar peptides, including the five already isolated (Donly *et al.*, 1993). Subsequently, it was shown that the homologous gene of *P. americana* encoded many of these same peptides, but had a total of 14 potential allatostatins in the primary sequence (Ding *et al.*, 1995).

The rather atypical hydrophobic nature and high tyrosine content of the octadecapeptide ASB2 (=allatostatin V=Dip-allatostatin 2=Dip-AST 2) of *D. punctata* led to the conjecture that it may have been representative of a different family of peptides (Pratt *et al.*, 1991). Molecular characterisation of the allatostatin neuropeptide precursors of *D. punctata* and *P. americana* have since indicated that this is an unlikely premise (Donly *et al.*, 1993; Ding *et al.*, 1995). Another unusual characteristic of this peptide is the presence of the dibasic cleavage site Lys^9-Arg^{10} within the internal N-terminal sequence. To date, the cleavage product Dip-AST 2 (residues 11–18) has not been identified in either *D. punctata* or in *P. americana*, although this octapeptide has been identified in the locust, *Schistocerca gregaria* (Veelaert *et al.*, 1995).

Table 1 *Cockroach allatostatins identified by isolation and purification*

Species	Designation[a]	Sequence[b]	Ref.[c]
D. punctata	Dip-AST 2, V, ASB2	A Y S Y V S E Y K R L P V Y N F G L-NH$_2$	[1]
	Dip-AST 4, VII	D G R M Y S F G L-NH$_2$	[2]
	Dip-AST 5, IV	D R L Y S F G L-NH$_2$	[3]
	Dip-AST 7, I	A P S G A Q R L Y G F G L-NH$_2$	[3,4]
	Dip-AST 8, III	G G S L Y S F G L-NH$_2$	[3]
	Dip-AST 9, II	G D G R L Y A F G L-NH$_2$	[3]
	Dip-AST 11, VI	Y P Q E H R F S F G L-NH$_2$	[2]
P. americana	Pea-AST 7, Pea-AST 1	S P S G M Q R L Y G F G L-NH$_2$	[5]
	Pea-AST 9, Pea-AST 2	A D G R L Y A F G L-NH$_2$	[5]
B. germanica	BLAST 1	L Y D F G L-NH$_2$	[6]
	BLAST 2	D R L Y S F G L-NH$_2$	[6]
	BLAST 3	A P S S A Q R L Y S F G L-NH$_2$	[6]
	BLAST 4	A G S D G R L Y S F G L-NH$_2$	[6]

Notes:

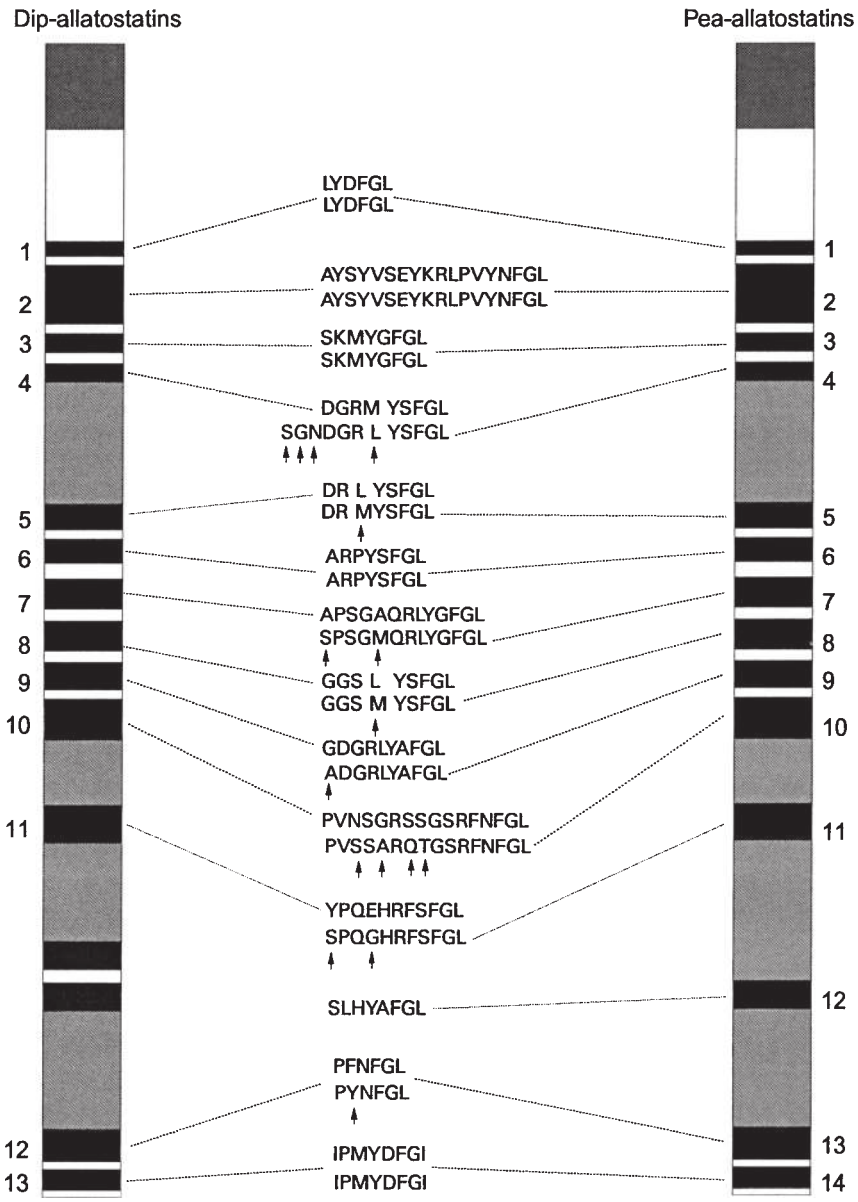
[a] Where more than one designation exists, the first cited designation is the one currently in use.

[b] Single-letter amino acid code.

[c] References: [1] Pratt *et al.* (1991); [2] Woodhead *et al.* (1994); [3] Woodhead *et al.* (1989); [4] Pratt *et al.* (1989); [5] Weaver *et al.* (1994); [6] Bellés *et al.* (1994).

Cockroach allatostatin genes

The amino acid sequence of the first allatostatin precursor was deduced from a cDNA sequence derived from mRNA isolated from the brains of virgin female *D. punctata* (Donly *et al.*, 1993; Stay *et al.*, 1994b). The specific cDNA encoding allatostatins was identified by polymerase chain reaction amplifications which used degenerate oligonucleotide primers designed by reverse-translation of purified *D. punctata* allatostatin peptide sequences (Woodhead *et al.*, 1989; Pratt *et al.*, 1991). DNA primers within the coding region of the *D. punctata* cDNA were subsequently used to amplify allatostatin-specific DNA fragments from both *P. americana* genomic DNA and brain-derived cDNA (Ding *et al.*, 1995). The coding regions for these cockroach preproallatostatin precursors are found as a single copy within the genome of each species, contained within approximately 1100 nucleotides and share 67% similarity at the nucleotide level. Genomic DNA sequences from both species are contiguous with cDNA in the region encoding the preproallatostatin precursor. However, introns have been found upstream of the initiator ATG. Conservation of DNA sequences outside of the coding region is limited but the (A+T)-rich character of the sequence 3′ to the coding region is conserved in both species. Surprisingly, the only detectable brain mRNA transcripts in *D. punctata* and *P. americana* are single species of 9.2 kb and 5.0 kb, respectively. Alternative mRNA species were not detected at different developmental times or in different tissues. Despite the size differences, features of AST mRNA expression appear to be conserved. AST mRNA has been localised by *in situ* hybridisation to several cells in the medial and lateral cell regions of the protocerebrum, as well as the tritocerebrum in both species. The mRNA size difference between the two species suggests that regulatory differences occur at the level of translation or mRNA stability of the mRNA transcripts. These differences may account, in part, for the differences in JH biosynthesis associated with the reproductive mode in each species (see below). The allatostatin polypeptide precursors of the two species share 71% amino acid sequence identity and are similar in structural organisation (Fig. 1). Each precursor is organised into domains beginning with a hydrophobic signal sequence domain required for translocation into the lumen of the endoplasmic reticulum, the allatostatin peptides with associated sequence Gly-Lys-Arg required for α-amidation and endoproteolytic cleavage, and finally acidic domains which probably serve to balance the basic charge contribution of the endoproteolytic cleavage sites. The *P. americana* precursor is cleaved into 14 different allatostatin peptides compared to the 13 peptides that are derived from the *D. punctata* precursor. The difference in peptide number is the result of a carboxyl-terminal substitution/deletion in the *D. punctata* counterpart to Pea-AST 12



which has destroyed the amidation signal that is required for biological activity. Dip-AST 1, 2, 3, 6, and 13 are identical to Pea-AST 1, 2, 3, 6, and 14 in both sequence and position in the precursor. Comparison of the remainder reveals that in the amino-terminal address sequences, conservative amino acid substitutions occur that maintain either the hydrophobic or aromatic character of the position. A notable exception is Pea-AST 4, which appears to have a three amino acid residue N-terminal extension relative to Dip-AST 4. These changes may provide an initial starting point for evolutionary grouping of other insect species at the genomic level.

Structure activity studies of cockroach allatostatins

Structure activity relationships for the *Diploptera* allatostatins, in terms of their ability to inhibit JH biosynthesis, have been determined, following the isolation and identification of the cDNA and the corresponding putative peptide precursor, with its 13 peptide products (see Stay *et al.*, 1994b; Bendena *et al.*, 1996; S. S. Tobe *et al.*, unpublished results). These data indicate a wide range of biological activities for the Dip-allatostatin family, with Dip-AST 2 as the most potent, showing an ED_{50} of 10 pM, followed by Dip-AST 5 with an ED_{50} of 100–200 pmol l^{-1} and Dip-AST 1 as the least potent, with an ED_{50} of *c.* 0.2 μmol l^{-1} (S. S. Tobe *et al.*, unpublished results). Thus, the Dip-allatostatins are active over four orders of magnitude. Interestingly, neither Dip-AST 2 nor 5 is particularly effective in modulating myotropic activity (ranking ninth and eighth of the 13 allatostatins, respectively) (Lange, Bendena & Tobe, 1995), indicating that the rank order for different biological activities, and presumably the interaction with receptors, depends upon the particular function under study.

Fig. 1. Schematic diagram of insect allatostatin polypeptide precursors deduced from isolated DNA sequences. The polypeptide precursors of cockroaches *Diploptera punctata* (left) and *Periplaneta americana* (right) begin with an NH_2-terminal hydrophobic leader region (cross-hatched) that is presumably cleaved by signal endoproteases. Black boxes represent the individual allatostatin peptides which are numbered according to their position relative to the amino-terminus in the precursor. The sequence of each allatostatin peptide is shown between the two precursors. The differences between the *D. punctata* and *P. americana* peptide sequences are indicated by arrows. Acidic regions are indicated by diagonal lines. Incorporated within the third acidic spacer region of the *Diploptera allatostatin* precursor are two non-amidated peptides (shaded boxes). Each allatostatin, from both species, precedes a Gly-Lys-Arg sequence required for endoproteolytic cleavage and α-amidation. Single-letter code for amino acids is used.

As noted below, the core pentapeptide of the allatostatins is highly conserved, with only residue 2 showing significant variability. The pentapeptide has significant efficacy and reduced, but appreciable, potency. As such, it probably represents the minimum necessary for binding to receptor(s), with modifications to the N-terminal address sequence altering potency but not efficacy (see Stay *et al.*, 1994b). Whether this translates into differences in binding affinity, to interaction with different receptor subtypes, to synergism with other allatostatin or other peptides, or to differences in ability to effect signal transduction following binding remains to be determined. Nonetheless, conservation of both structure and function for the core sequence suggests important biological activities for all of the allatostatins, including inhibition of JH biosynthesis and modulation of myotropic activity.

Structure activity studies have also recently been completed for the *P. americana* allatostatins. In summary, all 14 of the Pea-AST are capable of inhibiting JH III biosynthesis in CA from day 5 female *P. americana* in a dose-dependent manner, but with varying degrees of efficacy (Table 2). Maximal efficacy varied from high, for example, 80–90% for Pea-AST 1, 2, 3, 4, 6, 7, 9 to 55% for Pea-AST 5, whereas ED_{50} values showed a 400-fold range, from 0.7 nmol l^{-1} for Pea-AST 2 to greater that 0.3 μmol l^{-1} for Pea-AST 14.

The ED_{50} values for *D. punctata* and *P. americana* allatostatins show that length of the peptide does not correlate well with biological potency. One of the smaller of the *P. americana* allatostatins, the octapeptide Pea-AST 6, shows a potency only slightly lower (less than one order of magnitude) than that of Dip-AST/Pea-AST 2, the most active endogenous peptide in both species. The observed range of ED_{50} values may indicate that allatostatins of different length address sequences interact with different types of receptor (Stay *et al.*, 1994b). However, a comparison of the dose response data for the five *P. americana* octapeptides reveals that even short address sequence variations markedly influence the potency of the peptide. The address sequences of the *P. americana* allatostatins varies from 9–13 amino-terminal residues in the case of Pea-AST/Dip-AST 2 to 2–3 amino acid residues in the case of Pea-AST 3, 5, 6, 12 and 14. The least potent peptides tested were Pea-AST 5 and 14 which, even at the highest doses tested (10 μmol l^{-1}), were only capable of inhibiting JH biosynthesis by 50–60%. The data for Pea-AST 14 are not surprising, since this is the only allatostatin with an Ile residue at the important C-terminal position (see Stay *et al.*, 1994b). In contrast, the poor performance of Pea-AST 5 was unexpected, particularly since the equivalent Dip-AST 5 is one of the most active allatostatins when tested against *D. punctata* (Stay *et al.*, 1994b) and is also active both *in vivo* and *in vitro* in *P. americana* (Weaver *et al.*, 1995b). It would seem that the single amino