Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9458–9462, November 1991
Physiology

Identification of an allatostatin from the tobacco hornworm *Manduca sexta*

(juvenile hormone regulation/corpora allata/amino acid sequence)

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Communicated by Wendell Roelofs, July 29, 1991 (received for review May 24, 1991)

ABSTRACT **A peptide (*Manduca sexta* allatostatin) that strongly inhibits juvenile hormone biosynthesis *in vitro* by the corpora allata from fifth-stadium larvae and adult females has been purified from extracts of heads of pharate adult *M. sexta* by a nine-step purification procedure. The primary structure of this 15-residue peptide has been determined: pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe-OH, where pGlu is pyroglutamate). To our knowledge, this neurohormone has no sequence similarity with any known neuropeptide from other organisms. The synthetic free acid and amide forms showed *in vitro* activity indistinguishable from that of native *M. sexta* allatostatin. The ED_{50} of synthetic *M. sexta* allatostatin on early fifth stadium larval corpora allata *in vitro* was ≈2 nM. This inhibition was reversible. In a cross-species study, *M. sexta* allatostatin also inhibited the corpora allata of adult female *Heliothis virescens* but had no effect on the activity of corpora allata of adult females of the beetle *Tenebrio molitor*, the grasshopper *Melanoplus sanguinipes*, or the cockroach *Periplaneta americana*.**

Juvenile hormone (JH), which is synthesized and released by the retrocerebral corpora allata (CA), plays a vital role in insect development, primarily in the control of metamorphosis, adult sexual maturation, and reproduction (1, 2). Environmental and physiological factors influence neurosecretory centers in the brain that affect the activity of CA through peptidergic materials either transported directly to the CA by axons or released into the blood (3). These neuropeptides may be stimulatory (allatotropins, e.g., refs. 4–8) or inhibitory [allatostatins (ASs), e.g., refs. 9–17]. It has been widely acknowledged that the design of agents that antagonize and/or mimic the mode of action of insect neuropeptides could lead to environmentally sound insect control strategies (18, 19).

By using methodologies that have been instrumental in the isolation of a variety of mammalian neuropeptides, rapid progress has been made with the isolation of several insect neuropeptides (20). To date, several brain peptides that act on the CA have been identified. One is an allatotropin from heads of pharate adult *Manduca sexta* that stimulates the CA of adult lepidopterans only (21). Another is a family of four ASs [*Diploptera punctata* ASs (Dip-ASs) 1–4] from brains of the virgin female cockroach *D. punctata* (22, 23). The most potent Dip-AS, Dip-AS 1, inhibited JH synthesis *in vitro* of mated females and last-instar larvae of *D. punctata* and adult female *Periplaneta americana*.

In this report, we describe the purification, sequence analysis, and synthesis of a 15-amino acid peptide from the heads of pharate adult *M. sexta* that causes rapid and potent inhibition of JH synthesis in CA from larvae and adult female *M. sexta*.

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MATERIALS AND METHODS

Insects. *M. sexta* were reared on an artificial diet (24). Heads of pharate adult *M. sexta*, 24–48 hr before adult eclosion, were collected, frozen, and "trimmed" as described (25).

AS Bioassay. Throughout our purification procedure, we used an *in vitro* radiochemical method for assaying the ability of test samples containing AS to inhibit the secretion of JH from the CA (two pair per incubation) of 0–4 hr fifth-stadium *M. sexta* larvae. This method measures incorporation of the labeled moiety from L-[*methyl*-^{14}C]methionine into JH in the final step of its biosynthesis. We used the method of Pratt and Tobe (26) as modified for *M. sexta* (27). However, instead of assaying for JH production by chloroform extraction followed by TLC analysis, we used a rapid isooctane partitioning assay for JH (28). In the cross-species studies, we used the same *in vitro* technique (one or two pair of CA per incubation).

Extraction and Preliminary Purification (Steps 1–4). Thirty thousand trimmed heads of *M. sexta* (fresh weight, ≈1460 g) were processed in three batches of 10,000. Each batch was defatted by homogenizing in 2 liters of ice-cold acetone and filtered. Residues were extracted with 1.4 liters of 1 M HOAc/20 mM HCl (containing 0.1 mM phenylmethylsulfonyl fluoride and 0.01 mM pepstatin A, prepared immediately before use) and centrifuged at 10,000 × *g* for 30 min at 4°C. The pellet was extracted and centrifuged twice using a total volume of 2.8 liters of the same solution. The combined supernatants were stirred with swollen SP-Sephadex C-25 resin (300 ml) overnight. The resin was allowed to settle for 2 hr, subsequently poured into a Bio-Rad column (50 × 300 mm), and equilibrated with 1 M HOAc. Material was eluted from the column sequentially with 1-liter volumes of 0.05 M NH_4OAc (pH 4.0) and 0.05 M, 0.1 M, 0.2 M, 0.4 M, and 0.8 M NH_4OAc (pH 7.0). The 0.1 M and 0.2 M fractions, which had *M. sexta* AS (Mas-AS) activity, were applied directly to 10 g of reversed-phase Vydac C_4 packing material (20–30 μm, contained in a 75-ml polypropylene syringe barrel with a polyethylene frit) equilibrated with 0.1% trifluoroacetic acid (TFA). Material was eluted sequentially from the cartridge

Abbreviations: AS, allatostatin; Mas-AS, *Manduca sexta* AS; CA, corpora allata; JH, juvenile hormone; Dip-AS, *Diploptera punctata* AS; TFA, trifluoroacetic acid; LC, liquid chromatography; RCM, reduced carboxymethylated.

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with 100-ml volumes of 20, 40, and 60% (vol/vol) CH_3CN in 0.1% TFA. Mas-AS was recovered in the fractions of 40 and 60% CH_3CN in 0.1% TFA.

TSK SP-5PW Preparative Liquid Chromatography (LC) (Step 5). This and subsequent purifications by LC were performed with a Perkin–Elmer model 410 Bio pump, a Rheodyne loop injector, and Kratos model 783 variable wavelength detector, set at 220 nm. The pump was modified with a Rheodyne model 5302 valve installed in the D solvent tube before the solvent-proportioning valve, so that water-diluted fractions could be pumped into the column. The active fraction from step 4 was diluted 1:1 (vol/vol) with 0.1% TFA and pumped onto a TSK SP-5PW column (21.5 × 150 mm). Material was eluted from the column with a 90-min linear gradient of 0–0.5 M NaCl in 20 mM Mes buffer (pH 5.5) at a flow rate of 8 ml/min. Fractions of 12 ml were collected. Mas-AS activity was found in fractions eluting at 30–34 min.

Vydac C_4 Semipreparative LC (Steps 6 and 7). The active fractions from step 5 were combined, diluted 1:1 with 0.1% TFA, and pumped onto a Vydac C_4 semipreparative column (10 × 250 mm) previously equilibrated with water. Material was eluted from the column with a 60-min linear gradient of 20–26% (vol/vol) 1-propanol in 0.5% heptafluorobutyric acid at a flow rate of 5 ml/min; 10-ml fractions were collected. Mas-AS activity was present in fractions that eluted at 48–60 min.

Active fractions from the previous step were combined and diluted 1:2 with 1% TFA and pumped onto a Vydac C_4 semipreparative column previously equilibrated with 0.1% TFA. Material was eluted from the column with a 60-min linear gradient of 20–35% (vol/vol) CH_3CN in 0.1% TFA at a flow rate of 5 ml/min; 10-ml fractions were collected. Mas-AS activity was found in one fraction that eluted at 50–52 min.

TSK SP-5PW LC (Step 8). The active fractions from the previous step were diluted 1:1 with 1% TFA and pumped onto a TSK SP 5PW column (7.5 × 75 mm), equilibrated with 20 mM Mes (pH 5.5) containing 10% CH_3CN. Material was eluted from the column with a 30-min linear gradient of 0.2–0.5 M LiCl at a flow rate of 1 ml/min, in the same buffer. Fractions (2 ml) were collected. Mas-AS was recovered in the fractions at 26–28 min.

Vydac C_4 Microbore LC (Step 9). The active fraction from step 8 was applied to a Vydac C_4 microbore column (2.1 × 150 mm). The column was equilibrated with 10% 1-propanol in 0.1% TFA. After elution with a 60-min linear gradient of 10–25% 1-propanol/0.1% TFA at a flow rate of 0.2 ml/min, pure Mas-AS was recovered in a single peak at 41–42 min. This final step was performed on a Hewlett–Packard model 1090 LC.

Sequence Analysis. The terminal pyroglutamate residue of purified Mas-AS was removed with pyroglutamayl aminopeptidase (Boehringer Mannheim) at 5°C (29). The resultant 14-amino acid peptide was sequenced using an Applied Biosystems model 477A pulsed liquid-phase protein sequencer. Released phenylthiohydantoin amino acids were analyzed using an on-line analyzer (Applied Biosystems, model 120A).

Amino Acid Analyses. Purified Mas-AS was hydrolyzed in vapor from 6 M HCl/1% phenol (110°C, 20 hr). Amino acids in hydrolysates were analyzed as fluorescent derivatives with an AminoQuant (Hewlett–Packard). Native Mas-AS (170 pmol) was converted to the reduced carboxymethylated (RCM) derivative with tritiated iodoacetic acid (30). The amino acid composition of the RCM-Mas-AS was determined likewise.

Peptide Synthesis. Mas-AS was synthesized by the solid-phase method, using a Biosearch model 9600 peptide synthesizer and a *tert*-butoxycarbonyl (Boc)/diisopropylcarbodimide chemistry protocol. The amino acid side chains were protected in the following manner: Arg(tosyl), Cys^{14}(4-methylbenzyl), Cys^{7}(3-nitro-2-pyridinesulfenyl), Ser(benzyl), and Tyr(2-bromobenzyloxycarbonyl). Boc-Phe-*O*-resin and 4-methylbenzyhydrylamine resin were used to initiate the peptide sequence of the acid and amide forms, respectively. After HF cleavage in the presence of *p*-cresol, the crude peptide was converted to the disulfide form by stirring in 5% (vol/vol) acetic acid and the final peptide was purified by reversed-phase LC, using a C_4 column and isocratic elution with 33% CH_3CN/0.2% TFA at a flow rate of 1.5 ml/min.

Carboxyl Terminus Analysis. Synthetic Mas-AS and its corresponding carboxyl-terminal amide were reduced and carboxymethylated. The tritiated RCM derivative of native Mas-AS (17 pmol) was mixed with the above synthetic RCM standards (50 pmol each) and analyzed using a Hewlett–Packard model 1090 LC with UV detection at 220 and 280 nm. Material was eluted from a Vydac C_4 column (2.1 × 150 mm) at 0.3 ml/min with a gradient of 0–20% CH_3CN/0.1% aqueous TFA at 5–10 min, and then with 20–60% CH_3CN at 10–70 min.

RESULTS

Isolation Procedure. After lipids had been removed from the 30,000 trimmed pharate adult heads by extraction with acetone, Mas-AS was extracted with an acidic solution containing two protease inhibitors. This acidic solution was applied directly to an SP-Sephadex column, and material was eluted stepwise with various ammonium acetate buffers of increasing pH and/or ionic strength. The active fractions from SP-Sephadex were applied to a C_4 reversed-phase "cartridge" containing 10 g of adsorbent. Material was eluted from the cartridge stepwise with CH_3CN/0.1% aqueous TFA; Mas-AS-active material was eluted in the 40–60% CH_3CN fractions. The latter solution was diluted with water and then loaded onto a preparative TSK SP-5PW ion-exchange column by using the pump (31). Previously, we have demonstrated that this technique avoids losses of biological activity as opposed to when fractions are evaporated at each step (25). Subsequently, two successive semipreparative reversed-phase purifications were performed using different organic modifiers. The active fraction was further subjected to another ion-exchange LC (semipreparative) and Mas-AS was finally purified by microbore reversed-phase LC (Fig. 1). The overall yield was ≈1 nmol from 30,000 trimmed heads.

Structural Analysis. Initial automated Edman degradation studies of native Mas-AS indicated that it was an amino-terminal-blocked peptide. However, pyroglutamyl aminopeptidase treatment of reduced and [^{3}H]carboxymethylated Mas-AS gave a product that could be separated by

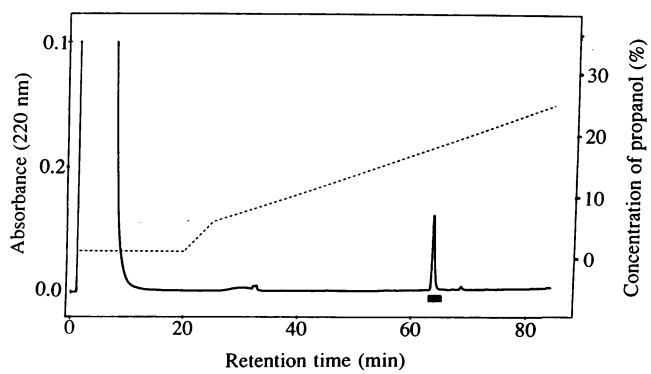

FIG. 1. Final separation of Mas-AS by reverse-phase LC. Bar indicates the area of AS activity. Solid line, absorbance; dashed line, propanol concentration.

Table 1. Amino acid composition analysis of Mas-AS

Amino acid	Native		RCM	
	Found	Best integral value	Found	Best integral value
Asx	1.4	1	1.1	1
Glx	3.0	3	2.1	2
Ser	2.1	2	0.9	1
His	0.4	0	0.1	0
Gly	2.0	2	0.4	0
Thr	0.7	1	0.3	0
Ala	0.9	1	0.4	0
Arg	2.2	2	1.9	2
Tyr	1.2	1	1.0	1
Val	1.0	1	0.9	1
Met	0.3	0	0.1	0
Ile	1.1	1	1.0	1
Phe	2.5	3	2.7	3
Leu	0.5	1 ?	0.2	0
Lys	0.5	1 ?	0.3	0
Pro	1.1	1	0.8	1
Cys		2	1.7	2

reversed-phase LC. Sequence analysis of this deblocked peptide yielded a single amino acid sequence of 14 residues with radioactivity in cycles 6 and 13.

Amino acid analysis of native Mas-AS resulted in an amino acid composition (Table 1), which exceeded the composition of the sequenced peptide. To resolve this apparent discrepancy, Mas-AS was reduced and the cysteine residues were carboxymethylated with tritiated iodoacetic acid. Amino acid analysis of the purified RCM peptide was totally consistent with the 15-amino acid peptide (Table 1).

The nature of the carboxyl terminus of MAS-AS was established by reversed-phase LC analysis of the RCM forms of tritiated native Mas-AS and synthetic carboxyl-terminal acid and amide (Fig. 2). Native RCM-Mas-AS was coeluted with the synthetic RCM acid when coinjected, as shown by elution of ^3H and peak enhancement of the RCM-Mas-AS standard. Thus, the complete structure of Mas-AS is pGlu-Val-Arg-Phe-Arg-Gln-Cys-Tyr-Phe-Asn-Pro-Ile-Ser-Cys-Phe-OH, where pGlu is pyroglutamate.

Biological Properties of Mas-AS. The biological activities of the synthetic free acid and amide forms were compared with native Mas-AS in the *M. sexta* CA *in vitro* bioassay. The potencies of the two synthetic forms were not significantly different from that of native Mas-AS (Fig. 3).

We tested the effects of Mas-AS on CA of other developmental stages of this insect and on CA of adult insects of four other species (Table 2). It is clear that Mas-AS is active only on the lepidopteran species.

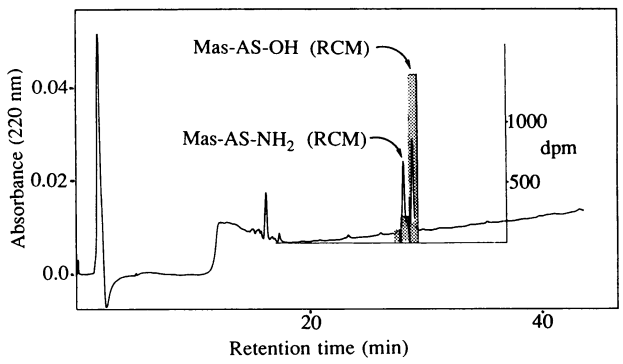

FIG. 2. Vydac C_{18} microbore LC of RCM synthetic peptides coinjected with ^3H-labeled RCM native AS. Synthetic peptides are either the free acid (Mas-AS-OH) or amidated (Mas-AS-NH_2) at the carboxyl terminus. The stippled histogram represents ^3H incorporated into the RCM derivative of native Mas-AS.

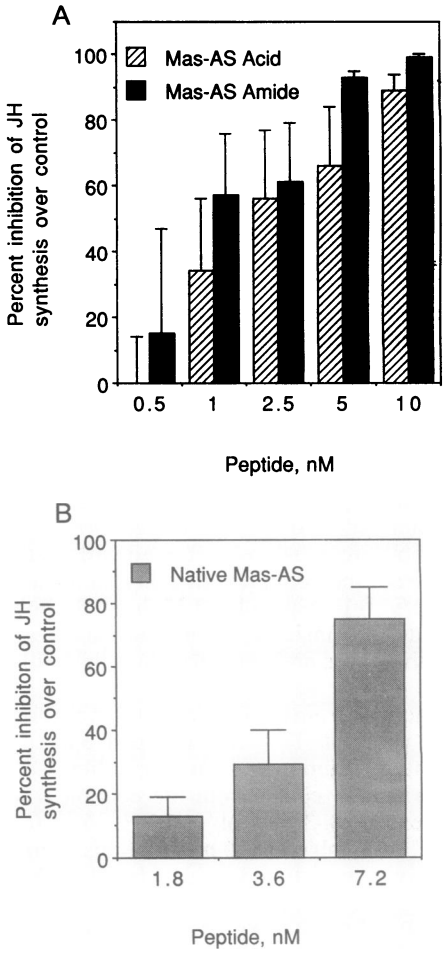

FIG. 3. (*A*) Dose–response of synthetic Mas-AS and its amidated analog (Mas-AS-amide) on JH biosynthesis by CA *in vitro*. Data bars represent mean values from 4 to 16 incubations. The error bars represent the SEM. (*B*) Dose–response of native Mas-AS on JH biosynthesis by CA *in vitro*. The quantity of Mas-AS tested was determined from the amino acid analysis. Data bars represent mean values from three incubations. The error bars represent the SEM.

DISCUSSION

We developed a nine-step purification procedure for the isolation of an AS, Mas-AS, from heads of pharate adults of *M. sexta*. This is the same stage and tissue that we used for isolation of the eclosion hormone (32), allatotropin (21), and the diuretic hormone (25), which were all sequenced in our laboratories. During this time, we also demonstrated the presence of AS activity in these head extracts by using an early-fifth-stadium *M. sexta* CA *in vitro* assay. Early attempts to isolate AS were unsuccessful because biological activity diminished during purification and was eventually lost, perhaps due to partially evaporating HPLC fractions between various isolation steps. During the isolation of *M. sexta* diuretic hormone (25), we had initially experienced the same problems. After modification of the pumping system so that water-diluted fractions containing organic solvent could be pumped onto the column, dramatic improvements in recovery were achieved. This technique was subsequently used for the purification of Mas-AS. In addition, to prevent oxidation of the peptide, we added the antioxidant 2-(methylthio)ethanol (0.1%) to fractions at each purification step. This reagent is volatile and can be removed from fractions dried down for bioassay.

Table 2. Effects of AS on CA activity *in vitro* in three orders of insects

Insect	Stage	Replicates, no.	JH synthesis, pmol per gland pair per hr		Mas-As dosage, pmol
			Control	Mas-AS	
Lepidoptera					
M. sexta	Adult ♀ (0–4 hr)	6	1.67 ± 0.31	0	10
Heliothis virescens	Adult ♀ (0–4 hr)	5	3.08 ± 0.69	0.71 ± 0.51	50
Orthoptera					
P. americana	Adult ♀ (dark oothecae)	7	9.89 ± 3.44	8.50 ± 3.05	50
Melanoplus sanguinipes	Adult ♀ (7 days old)	4	6.90 ± 4.36	10.49 ± 4.46	50
Coleoptera					
Tenebrio molitor	Random adults	5	6.91 ± 0.81	5.15 ± 1.40	100

Data for JH synthesis are mean ± SEM.

Eclosion hormone (32), allatotropin (21), and diuretic hormone (25) of *M. sexta* were isolated using variations of a similar procedure, requiring only eight or nine steps. For the isolation of Mas-AS, we followed basically the same strategy. In addition, we included a preparative ion-exchange LC step before the reversed-phase LC purification steps. This step was very useful because of its high capacity and resolution power, important because we used three times as much tissue compared with our other hormone isolations (21, 25, 32). A crucial difference from the isolation procedure of the other three hormones was the use of 1-propanol/0.5% heptafluorobutyric acid for the first reversed-phase LC step. Pilot studies showed that no other solvent system we have used (21, 25, 32) could resolve a large peak of contaminants (apparently structural proteins) from the Mas-AS. In step 8 (TSK SP-5PW LC), we used LiCl (a weaker eluent than the sodium ion) for the eluting gradient to provide better selectivity in the separation.

The amount of AS recovered was about 0.033 pmol per head, ≈20% of the amount of eclosion hormone (0.17 pmol per head; ref. 32) or allatotropin (0.15 pmol per head; ref. 21) recovered from the same source and only ≈7% of the amount of diuretic hormone (0.5 pmol per head; ref. 25). Unfortunately, due to the nature of the bioassays, we have no estimate of the comparative recovery of biological activity in each case. Because the isolation schemes used are similar, we would presume that the chemical recoveries would also be similar and that these numbers would be representative of the amounts of each hormone *in vivo*. However, we did not use antioxidant during the isolation of diuretic hormone and allatotropin, both of which contain two methionine residues, which are notoriously prone to oxidize during isolation, usually to less biologically active products (33). Thus, the recoveries of these two hormones may actually have been relatively less than for Mas-AS. In addition, Mas-AS may be released down axons to the CA, rather than directly into the blood like eclosion hormone or diuretic hormone, which are believed to act on more distal tissues. In acting on a more proximal target organ, perhaps less AS is required. Moreover, at this developmental stage there is no reason to expect much AS in *M. sexta*; on the contrary, there should be allatotropin because the CA start secreting JH at adult emergence (1, 2).

Amino acid analyses of native Mas-AS did not agree with results from sequence analysis (23 amino acids instead of 15 amino acids). This discrepancy was solved by reduction and carboxymethylation of the peptide and subsequent amino acid analysis of the purified product, which gave results consistent with a 15-amino acid composition. In addition, RCM-Mas-AS coeluted with the synthetic 15-residue RCM acid form when coinjected. The additional amino acids found on analysis of native Mas-AS may have been contributed by a coeluting peptide(s) present in lesser amounts. Sequencing of several trace peptides isolated from RCM deblocked material suggested structures dissimilar to Mas-AS. Thus, none of these impurity peptides is expected to be an allatostatin.

We checked for sequence similarity of Mas-AS with known peptides by using an IntelliGenetics protein data base (which accessed the National Biomedical Research Foundation Protein Sequence Data Bank on April 2, 1991). No significant sequence similarity was found with any known peptide or protein.

Dose–response studies with native Mas-AS and the synthetic acid and amide forms on young fifth stadium *M. sexta* CA *in vitro* resulted in a similar range of ED_{50} values of 2–5 nM for all three peptides. This range is the same as that found for the most active AS (Dip-AS 1) isolated from the cockroach *D. punctata* (22, 23). The finding that natural Mas-AS and the corresponding synthetic amide form possess virtually identical bioactivity is a rare phenomenon among peptides. Another such example is mammalian cecropin P1, in which natural acid and synthetic amide forms have equal antibacterial activity (34).

The inhibition with Mas-AS is completely reversible; transfer of inhibited CA to fresh medium results in restoration of the gland activity (A.T. and S.J.K., unpublished data). This is in agreement with results previously shown for Dip-AS 1 and Dip-AS 2 (22).

In another study using *D. punctata* (23), it was concluded that Dip-AS 1 exerts its action in the JH biosynthetic pathway prior to the conversion of farnesol to JH. We also found this to be likely for Mas-AS, because coincubation of Mas-AS and the JH biosynthetic intermediate farnesoic acid (20 μM) resulted in a "rescue" effect (A.T. and S.J.K., unpublished data). Biological studies in which Mas-AS was tested *in vitro* on CA of adult insect species (Table 2) demonstrated that Mas-AS is only active on CA of lepidopterans. The lack of activity in cockroaches was not surprising since Mas-AS shows no sequence homology with the four ASs from *D. punctata* (22), which, moreover, do not contain disulfide bonds. Unlike the multiple ASs of *D. punctata*, we found no evidence for the existence of more than one AS in *M. sexta*.

Analogues of JH are currently used for control of a number of insect species in which the adult is the primary pest (35) but are not useful for controlling crop-destroying insects. Interference with JH biosynthesis, a complicated cascade of biochemical events (36), has been extensively studied (37, 38). So far, anti-JH agents that would be useful in controlling crop-destroying insect larvae have lacked sufficient potency necessary for commercialization (37, 38). The availability of the structure of a lepidopteran AS will now facilitate studies on the mode of action of JH-controlling agents at the cellular and enzymatic level. This should lead to the design of chemicals that interfere with JH biosynthesis.

We thank the staff of the insect culture group for providing insects; Marilyn Bennett, Quyen Nguyen, and Lina Summerfelt for technical assistance; and Steve Bowlus for a computer search of peptide homology.

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