Callatostatins: Neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to cockroach allatostatins

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**ABSTRACT**

Five neuropeptides with C-terminal amino acid sequence homology to cockroach allatostatins have been identified in the blowfly *Calliphora vomitoria*. Three have the same pentapeptide C-terminal amino acid sequence as allatostatin I of the cockroach *Dipterora punctata*. A hexadecapeptide designated callatostatin 1, isolated from thoracic ganglia, brains, and heads, has the sequence Asp-Pro-Leu-Asn-Glu-Glu-Arg-Arg-Ala-Asn-Arg-Tyr-Gly-Phe-Gly-Leu-NH$_2$. Callatostatins 2 and 3 have been isolated from heads and thoracic ganglia, respectively; they comprise the last 14 and 8 residues of callatostatin 1. Callatostatin 4, isolated from thoracic ganglia, has the sequence Xaa-Arg-Pro-Tyr-Ser-Phe-Gly-Leu-NH$_2$, where Xaa is either Asp or Asn. This peptide, with a serine substitution for glycine at position 5, has a C-terminal pentapeptide sequence identical to that of allatostatins 3 and 4 of *D. punctata*. Callatostatin 5, with the sequence Gly-Pro-Pro-Tyr-Asp-Phe-Gly-Met-NH$_2$, was identified from whole flies. All five peptides inhibit juvenile hormone production by the corpora allata of *D. punctata* in vitro. Callatostatin 5 was the most potent allatostatin so far tested in this species, with maximum inhibition occurring at 1 nM. In contrast, none of the callatostatins or the allatostatins showed allatostatic activity in mature female *C. vomitoria* when tested at concentrations of 100 to 0.1 μM. In accordance with these results, immunoreactivity to an antiserum directed against the common C terminus of callatostatin 1 and allatostatin 1 was observed in the corpora allata of *D. punctata* but not in the corpus allatum of *C. vomitoria*, despite its presence in neurons of the brain. Neurons in the thoracic ganglion of *C. vomitoria* that are immunoreactive against this antiserum project to the hindgut, rectum, rectal papillae, and oviduct, suggestive of a function different from that of a true allatostatin.

Neuropeptides capable of inhibiting the production of juvenile hormone by the corpus allatum of insects have been termed allatostatins (1). To date, members of this class of neuropeptides have been identified in only two species. In the cockroach *Dipterora punctata*, five allatostatins ranging in size from 8 to 18 amino acid residues have been characterized (2-4). These neuropeptides show C-terminal similarity, which suggests that they are members of a family, possibly originating from a single gene. From the lepidopteran *Manduca sexta*, an N-terminally blocked allatostatin with an amino acid sequence different from those of the cockroach has recently been identified (5).

We report here on the identification, structure, localization, and biological activity of five neuropeptides from the blowfly *Calliphora vomitoria* with sequence homology to the cockroach allatostatins.

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**EXPERIMENTAL PROCEDURES**

*Insects and Tissue Extraction*. Adult *C. vomitoria* maintained at 25°C and 65% relative humidity were fed a diet of sugar, water, and protein (either Ovaltine or liver). Sources of material and extraction procedures were as follows; dissected thoracic ganglia (*n* = 3625), 80% methanol/0.1 M HCl/0.1% 2-mercaptoethanol (6); dissected brains (*n* = 1000), boiling 0.9% NaCl (2); heads (*n* = 20,000), 87% methanol/5% acetic acid/8% water followed by acetone precipitation (7); whole flies (*n* = 10,000), 1 M acetic acid/0.02 M HCl/0.1% 2-mercaptoethanol (8).

*Chromatography*. A summary of the chromatographic procedures for the purification of callatostatins from heads, thoracic ganglia, and whole flies is given in Fig. 1. For brains, the protocol of the first three HPLC steps has been published previously (9). A final step added to the procedure was identical to that given in the protocol for the purification of the peptides from whole flies (Fig. 1). The initial purification of callatostatin 1 from thoracic ganglia was as described in a study of the *Calliphora* -Phe-Met-Arg-Phe-NH$_2$ peptides (the *callFMR* Family; ref. 6).

*Radioimmunoassay*. Column eluants (10-200 μl) of each fraction were monitored by means of a RIA using a polyclonal antiserum specific for callatostatin 1 and 125I-labeled synthetic callatostatin 1 (Core Facility, Insect Biotech Canada, Kingston, ON). The basic details of the RIA protocol are identical to those described earlier for YGGFMRF (8). The assay requires the carboxyamidated *hexapeptide* C-terminal amino acid sequence of callatostatin 1 for full recognition. Thus, allatostatin 1 from the cockroach was detected at a level of only ~10%, despite the fact that the C-terminal *pentapeptide* sequence is identical to that of callatostatin 1. With allatostatins 2-4, where substitutions with respect to allatostatin 1 occur at the fourth residue from the C terminus, recognition in the assay was <1%. Callatostatin 5, in which the C-terminal leucine residue is replaced by methionine, and nonamidated callatostatin 3 were not detected by this RIA.

*Amino Acid Sequence Analysis*. The amino acid sequences of the purified peptides (5-50 pmol) were determined with an automatic protein sequencer (model 475A, Applied Biosystems) equipped with an on-line HPLC system for the detection of the amino acid phenylthiohydantoin derivatives, which were separated on a C18-DB column (5-7943, Supelco). All chemicals and solvents were sequence or HPLC grade (Applied Biosystems).

*Mass Spectrometry*. A portion (5-50 pmol) of each of the purified peptides was analyzed by using a Bio-Ion 20 plasma desorption time-of-flight mass spectrometer (Applied Biosystems). The dried samples were dissolved in 10 μl of 0.1% trifluoroacetic acid in 20% acetonitrile and two 5-μl aliquots

Abbreviations: JH$_B$, juvenile hormone III bisepoxide; JH III, juvenile hormone III.

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were applied to aluminized Mylar foil (coated with nitrocellulose) and evaporated. The spectra were recorded for 1–6 × 10^6 primary ions. The method has an accuracy of 0.1%. For callatostatin 1, the number of free carboxyl groups was determined by subjecting an aliquot to methylation and remeasuring the mass. The method requires a 2-hr reaction of the peptide with 2 M HCl in methanol, obtained by addition of acetyl chloride to dry methanol (10).

**Peptide Synthesis.** Peptides were synthesized on an Applied Biosystems model 430A automatic solid-phase peptide synthesizer (Insect Biotech Canada). They were purified on a preparative C_{18} reversed-phase column (Vydac).

**Assays for Allatostatic Activity.** The corpus allatum of C. vomitoria produces juvenile hormone III bisepoxide (JHB_{III}). Details of the radiochemical assay used to measure the rates of biosynthesis and release of this compound have been published previously (9), as have details of the assay used to measure the rates of production of juvenile hormone III (JH III) in D. punctata (2, 11–13).

**Immunocytochemistry.** For immunocytochemistry, aqueous Bouin’s fixative was used (14, 15). Paraffin sections (6 μm) were obtained from the brain, corpus allatum, and thoracic ganglion of 7-day-old female C. vomitoria flies fed sugar, meat, and water, as well as from the brain and corpora allata of 2-day-old virgins or 7-day-old mated D. punctata. They were immunostained with two antisera specific for the common C terminus of callatostatin 1 and allatostatin 1 by using the peroxidase-antiperoxidase technique (16). For whole mounts, the indirect immunofluorescence technique was used (17). Controls included liquid-phase absorption of the antisera with callatostatin 1 or allatostatin 1. Results showed conclusively that both peptides (10 nM) were able to abolish the immunostaining of the two antisera (diluted 1:1000).

**RESULTS**

**Peptide Identification.** A total of five peptides, designated callatostatins 1–5, were isolated in this study (Fig. 2). In addition, partial amino acid sequences and/or molecular weights were recorded for two other different callatostatin-immunoreactive peptides (not further discussed).

The hexadecapeptide callatostatin 1 (Fig. 2) was initially identified during the purification of a series of N-terminally extended -FMRFamide peptides (the calliFMRFamides) from thoracic ganglia of C. vomitoria [22.2% CH_{3}CN, step 5 (6)]. In the present study, the same peptide was also obtained from extracts of heads and brains monitored with the subsequently developed RIA for callatostatin 1 (Figs. 1 and 3). After methylation of an aliquot of callatostatin 1 obtained from thoracic ganglia, a molecular weight of 149.1 was recorded. This compares with an expected molecular weight of 1948.2 with free carboxyl groups on the three acidic amino acids, Asp^{4}, Glu^{5}, and Glu^{6}, but not the C-terminal Leu^{16}. The conclusion both from this experiment and from the results of the RIA, which is specific for the amidated C terminus, is that callatostatin 1 is amidated.

Also obtained from heads and identified by means of callatostatin 1 RIA was the truncated tetradecapeptide callatostatin 2 (Fig. 2). The truncated octapeptide, callatostatin 3, also immunoreactive in the callatostatin 1 RIA, was obtained from thoracic ganglia. Insufficient amounts of callatostatin 2 or 3 were available for methylation. However, from the results of RIA compared with semiquantitative

![Fig. 1](image-url). Summary of chromatographic procedures for the isolation of callatostatins from C. vomitoria. Trifluoroacetic acid (TFA) was used at 0.1%, and ammonium acetate (NH_{4}OAc) was used at 10 mM, pH 6.5. Purification of this material (steps 3–5, ref. 6) yielded callatostatin 1.
results from sequence analyses, we conclude that these peptides are C-terminally amidated.

The octapeptide callatostatin 4, where the residue at position 1 is either asparagine or aspartic acid, was also obtained from thoracic ganglia. This peptide was only weakly immunoreactive in the callatostatin 1 RIA, and we cannot be certain that it is carboxyamidated. However, the fact that the C-terminal pentapeptide sequence of this peptide is homologous with those of the cockroach allatostatins 3 and 4 makes it is likely that the posttranslational amidation has been conserved.

Callatostatin 5 was obtained from whole flies (Figs. 1 and 2) (8). This peptide was the major sequence of a doublet in a prominent UV peak which showed weak immunoreactivity in RIA against YGGFMRF. The minor sequence of this pair (incompletely identified) was probably the cause of the YGGFMRF immunoreactivity.

Assays for Allatostatic Activity. Callatostatins 1–4, synthesized in the amidated form, and callatostatin 5 in both amidated and free acid forms, were tested for allatostatic activity in both C. vomitoria and D. punctata according to published methods (9). Callatostatins 1–5, at selected concentrations from 100 to 0.1 μM, were inactive on the corpus allatum of C. vomitoria (6- to 10-day-old female flies in which the first gonadotrophic cycle was advanced or complete). The rate of production of JHB3 remained unchanged over a 3-hr test period relative to controls. In contrast, JH III biosynthesis in the cockroach was inhibited with each of the callatostatins, the most potent peptide being callatostatin 5 (IC50 = 0.1 nM) (Fig. 4). Callatostatin 5 in the free acid form was inactive on the corpora allata of C. vomitoria even at 100 μM and showed only a slight inhibition on the corpora allata of D. punctata at 100 nM.

Immunocytochemistry. Immunocytochemical studies have identified callatostatin 1 and allatostatin 1 immunoreactivity in perikarya in the brain—suboesophageal ganglion of both C. vomitoria and D. punctata, as well as in axons and arborizations in the neuropil (Fig. 5). The patterns of immunostaining were identical with these two antisera and the results shown here were obtained with callatostatin 1 antiserum. In D. punctata, (Fig. 5E), but not in C. vomitoria (Fig. 5A), callatostatin 1-immunoreactive nerve terminals were observed in the corpora allata. Immunoreactive perikarya and arborizations in the neuropil were also observed in the thoracic ganglion of C. vomitoria (Fig. 5B). A group of neurons in the abdominal ganglion projected axons posteriorly.
orly to the hindgut, rectum, rectal papillae, and oviduct (Fig. 5 B and C). In D. punctata, lateral neurosecretory cells of the brain showed strong callatostatin 1 immunoreactivity in 7-day-old mated females (Fig. 5D) but weak immunoreactivity in 2-day-old virgins. In contrast, immunoreactive cells of the median neurosecretory group appeared the same in animals of both ages (data not shown).

**DISCUSSION**

In this study we have identified five peptides from the blowfly C. vomitoria with sequence homology to the allatoxins of the cockroach D. punctata (2–4). The peptides have been designated callatostatins to take into account (a) the species of origin [also in line with the recent calliFMRFamide designation (6)], (b) the apparent sequence homology with the cockroach allatoxins, and (c) their ability to inhibit JH III production in the cockroach. Callatostatins 1–5 were isolated from thoracic ganglia, brains, and heads, as well as from whole flies. The C-terminal pentapeptide sequence -Tyr-Gly-Phe-Gly-Leu-NH₂ of callatostatins 1–3 is identical with that of allatoxin 1 from D. punctata. Callatostatin 4 shares with allatoxins 3 and 4 the substitution of serine for glycine at position 4 from the C terminus. Callatostatin 5 is different from any of the cockroach allatoxins identified to date in having methionine instead of leucine at the C terminus and aspartic acid at position 4 from the C terminus. We were unable to show whether the methionine is amidated, although bioactivity studies with both free acid (negative) and amidated form (strongly positive) suggest that it is. The residue at position 6 from the C terminus in allatoxins 1–4 is leucine, whereas in callatostatins 1–3 it is arginine and in callatostatins 4 and 5 it is proline. This residue appears to be variable even in the cockroach, since ASB2 (allatostatin 5) has valine in this position (4).

An interesting feature of callatostatin 1 is the presence of a pair of arginine residues at positions 7 and 8. A similar potential dibasic cleavage site (-Lys-Arg-) occurs at positions 9 and 10 of the cockroach octadecapeptide allatoxin 5 (4). In C. vomitoria, we have isolated the octapeptide (callatostatin 3) that would result from such prohormone processing. However, this sequence may also be coded for separately on the callatostatin gene. Furthermore, we have identified callatostatin 2, which lacks the first two N-terminal residues, Asp-Pro-. This peptide could originate from posttranslational cleavage of the prohormone, but it could also represent a distinct amino acid sequence determined by the gene, or an artifact of the extraction procedure (18).

All callatostatins have the ability to inhibit JH III release when tested on corpora allata of 2-day-old virgins of D. punc-
tata. The most potent peptide so far tested in this species is, in fact, callatostatin 5, not yet identified in *D. punctata* itself. Of the other peptides, callatostatins 1 and 2 were more potent than the shorter callatostatin 3, a finding in agreement with structure–activity studies of the cockroach allatostatins (4).

Although peptides with allatostatic activity have recently been shown to exist in partially purified brain extracts of *C. vomitoria* (9), none of the callatostatins 1–5 identified in the present study (and none of the cockroach allatostatins 1–5) was capable of inhibiting the production of JHB4 from the corpus allatum of *C. vomitoria* under our assay conditions. This suggests either that the callatostatins are important in the inhibition of JHB3 production during other stages of the life cycle (e.g., in larval stages or during adult diapause) or that they serve a different function. Evidence for the latter has come from immunocytochemical studies of the central nervous system and retrocerebral complex of *C. vomitoria* using a C-terminal-specific callatostatin 1 antiserum. Axons of the callatostatin 1-immunoreactive neurons in the median neurosecretory cell group do not appear to project to the corpus allatum. Furthermore, the lateral neurosecretory cells, another known source of innervation of the corpus allatum, show no immunoreactivity to the callatostatin antiserum. In contrast, the corpora allata of the cockroach are infiltrated by callatostatin 1-immunoreactive material, presumably within nerve terminals of axons originating from the median or lateral neurosecretory cells. Of particular interest is the fact that the callatostatin 1-immunoreactive neurons of the thoracic ganglion of the blowfly directly innervate the hindgut, rectum, rectal papillae, and oviduct. These neurons do not appear to transport any of their material to the dorsal neural sheath for release into the hemolymph. Thus, it appears unlikely that the callatostatins so far identified affect the corpus allatum, even indirectly.

In conclusion, it is clear that despite a conservation of amino acid sequence within the "allatostatins" in two widely divergent groups of insects, a conservation of function of the peptides—i.e., as inhibitors of juvenile hormone—is not apparent.

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