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Degradation of pheromone biosynthesis-activating neuropeptide (PBAN) by hemolymph enzymes of the tobacco hornworm, *Manduca sexta*, and the corn earworm, *Helicoverpa zea*

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Abstract. The tritium-labeled bis-norleucine analog of *Helicoverpa zea* pheromone biosynthesis-activating neuropeptide ([^{3}H]NLPBAN) was incubated in vitro with hemolymph from *Manduca sexta* or *H. zea* adult females. The incubations resulted in the formation of several tritium-labeled degradation products. At a [^{3}H]NLPBAN concentration of 0.9 µM the degradation proceeded at a very slow but physiologically plausible rate (2–10 fmol/min/µl hemolymph). The primary [^{3}H]NLPBAN degradation reaction in *M. sexta* hemolymph was not inhibited by 20 µM leupeptin, 0.1 mM amastatin, 1 mM EDTA, 1 mM EGTA, 1 mM 1,10-phenanthroline, or 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride; but secondary reactions may have been affected, as some of the inhibitors changed the radio-HPLC profile of the degradation products. It is concluded that hemolymph of *M. sexta* and *H. zea* contains peptidase(s) capable of inactivating circulating PBAN.
Key words. Bis-norleucine Hez-PBAN; *Manduca sexta*; *Helicoverpa zea*; hemolymph peptidases.

The control of pheromone production by a neuropeptide from the sub-oesophageal ganglion of an insect was first reported for adult females of the corn earworm, *Helicoverpa (Heliothis) zea*[1]. The pheromone biosynthesis-activating neuropeptide of *H. zea* (Hez-PBAN) and its gene have been isolated and the sequences have been determined[2,3]. Hez-PBAN is a 33-residue peptide with an amidated C-terminal leucine and two internal methionine residues[2]. Similar PBANs have been isolated from *Bombyx mori* (Bom-PBAN-I and II)[4] and from *Lymantria dispar* (Lyd-PBAN)[5], each with about 80% sequence homology to Hez-PBAN.

Pheromonotropic activity has been reported for several other insect species (refs in 6, 7). Hez-PBAN is active in several species of moths, including *Manduca sexta*[2] and, conversely, brain plus sub-oesophageal ganglion extracts of several species of moths have shown pheromonotropic activity when tested in *H. zea*[7]. Injection of Bom-PBAN-I into *Spodoptera litura* elicits different responses depending on the developmental stage treated. In larvae, it acts as a melanization and reddish coloration hormone; in adults, as PBAN[9]. The C-terminal pentapeptide of PBAN is essential for the pheromonotropic activity[8], and in fact has significant biological activity by itself[4,8]. The pentapeptide has a high degree of homology with the C-terminal sequence of myotropic peptides isolated from locusts and cockroaches. Pheromonotropic and myotropic peptides are highly cross-reactive[4,7].

Two routes have been proposed for PBAN transport from the sub-oesophageal ganglion to the pheromone gland: neuronal transport to the corpora cardiaca and release into the hemolymph[7], or neuronal transport via the ventral nerve cord to the terminal abdominal ganglia[10]. Although this is still a matter of some uncertainty, evidence is accumulating that at least in some species PBAN reaches the pheromone gland via the hemolymph[6]. The glands do respond to PBAN-containing hemolymph, because PBAN is effective when injected into the hemocoel[1,2,4] or when fed to female moths[11]. These observations raise the question of whether PBAN is stable in hemolymph or is degraded by hemolymph enzymes. Hemolymph of the cockroach *Periplaneta americana*[12,13] and of *M. sexta*[12] larvae contains peptidases that hydrolyze the myotropic neuropeptide proctolin in vitro. Hemolymph of the face fly *Musca autumnalis* appears to have peptidase(s) capable of degrading an adipokinetic and a hypotrehalosemic neuropeptide[14]. A hemolymph endopeptidase in *M. sexta* larvae, pupae and adults was found to hydrolyze various peptides of the adipokinetic hormone (AKH) family[15], but no such activity was detected in hemolymph of *Schistocerca gregaria*[16,17]. The C-terminal hexapeptide of PBAN was reported to be hydrolyzed by in vitro incubation with hemolymph of adult *B. mori* females, presumably as a result of aminopeptidase action[18].

The aim of the present study was to determine whether PBAN is similarly susceptible to hemolymph peptidases. The bioactive bis-norleucine analog (Nle^{5,14}) of Hez-PBAN (NLPBAN)[19] was chosen as a model sub-

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strate to avoid complications arising from the oxidation of the methionine residues in PBAN[2,4], and tritium labeling was used to allow monitoring of reactions at very low substrate concentrations. A previous communication dealt with the parameters for reproducible quantification of nanomole amounts of [^{3}H]NLPBAN[20]. Here we report the first evidence for in vitro degradation of PBAN by insect hemolymph enzymes and describe the effects of peptidase inhibitors on this process.

Materials and methods

NLPBAN, a 33-residue C-terminally amidated peptide of molecular weight 3,900 (Leu-Ser-Asp-Asp-Nle-Pro-Ala- Thr- Pro- Ala- Asp- Gln- Glu- Nle- Tyr- Arg- Gln-Asp- Pro -Glu- Gln- Ile- Asp- Ser- Arg- Thr- Lys- Tyr-Phe- Ser- Pro- Arg- Leu- NH_2), was obtained by catalytic tritiation or hydrogenation of iodo-NLPBAN ($I_2Tyr^{15,28}$-NLPBAN) to yield tritiated or unlabeled NLPBAN, respectively. Iodo-NLPBAN was synthesized on a Millipore 9600 peptide synthesizer (Milford, MA, USA) using the Fmoc protocol. Protecting groups were: PMC (Arg), Tmob (Gln), tBu (Asp, Glu, Ser, and Thr), and Boc (Lys). The Fmoc-3,5-diiodotyrosine was obtained from Bachem Bioscience (Philadelphia, PA, USA), and the other protected amino acids were from Millipore or Peninsula Laboratories (Belmont, CA, USA). The synthesis followed the manufacturer's protocols for coupling with diisopropylcarbodiimide and 1-hydroxybenzotriazole onto the peptide amide linker resin. After drying in vacuo overnight, the resin was cleaved with trifluoroacetic acid (TFA)/triethylsilane/water (90:5:5 by vol.), and the solution was rotary-evaporated and lyophilized to give a fluffy powder, which was purified by reversed-phase HPLC on a 20 × 250 mm C_{18} column (YMC Corp., Wilmington, NC, USA) using a 20–70% (vol.) acetonitrile/water gradient containing 0.05% TFA. Iodo-NLPBAN (10 mg) was dissolved in water, 10% Pd/C (10 mg) was added along with a few drops of triethylamine, and the material was hydrogenated on a Parr apparatus (Parr, Moline, IL, USA) for 30 min. After filtration and lyophilization of the peptide, the correct molecular weight for NLPBAN was confirmed by electrospray mass spectrometry. [^{3}H]NLPBAN was prepared by catalytic tritiation of Iodo-NLPBAN (Du Pont NEN Research Products, Boston, MA, USA). [^{3}H]NLPBAN, unlabeled NLPBAN, or mixtures thereof was purified by HPLC and measured as reported previously[20]. Peptidase inhibitors were obtained from Sigma (St. Louis, MO, USA).

Tobacco hornworms (*M. sexta* L.) were reared as described previously[21]. *H. zea* were reared from eggs obtained from the USDA Laboratory in Tifton, GA, USA, as described previously[22]. Adult female *M. sexta* were anesthetized by carbon dioxide and bled by decapitation into ice-cooled glass tubes containing a small volume (0.5–1.0 ml) of buffer (68 mM potassium phosphate, pH 7.5, 0.01% phenylthiourea) and a few additional crystals of phenylthiourea. *H. zea* hemolymph was obtained as described previously[1]. Hemolymph samples were kept at −83 °C until used. After thawing of the frozen hemolymph samples, more buffer was added to obtain 1/2 dilution of the hemolymph samples. The samples were centrifuged for 15 min at 16,000 *g* and 5 °C. Supernatants were either used directly (*M. sexta*, *H. zea*) or further processed by gel filtration or dialysis (*M. sexta*). Gel filtration was performed on a 2.5 × 13 cm Sephadex G-25 (fine; Pharmacia Biotech, Piscataway, NJ, USA) column with 34 mM potassium phosphate buffer, pH 7.5, and the protein fractions were pooled and used for enzyme assays. Other samples were dialyzed in 12,000–14,000 molecular weight cut-off tubing (Cellu.Sep T3, Membrane Filtration Products, San Antonio, TX, USA) for 17 h at 5 °C against 34 mM potassium phosphate buffer, pH 7.5. Protein concentrations were determined according to the Folin microassay of Peterson[23].

A stock solution of [^{3}H]NLPBAN (0.145 µg/µl; diluted to 30 Ci/mol with unlabeled NLPBAN) was prepared in a mixture of water/ethanol/acetic acid (70:30:0.1 by vol.) containing 10 mM mercaptoethanol. Aliquots of this stock solution (3.6 µl) were transferred into small volumes (15–50 µl) of 34 mM potassium phosphate buffer, pH 7.5, in polypropylene microcentrifuge tubes, followed by addition of hemolymph preparation or buffer (for controls). Incubation mixtures contained 133 pmol (0.9 µM) [^{3}H]NLPBAN, and 0.8–2.0 mg hemolymph proteins in a total volume of 150 µl. For inhibitor experiments hemolymph samples were incubated with the inhibitor for 15 min at 30 °C before addition to the buffer-[^{3}H]NLPBAN mixtures. Incubations were run for 2–4 h at 30 °C and terminated by addition of either an equal volume or a 9-fold excess of methanol. Samples were kept at −83 °C until analyzed.

In preparation for analysis, samples were centrifuged for 15 min at 16,000 *g* and 5 °C. Supernatants of samples containing 50% methanol were acidified with TFA, aliquots were taken for liquid scintillation counting, and the samples were analyzed by HPLC. Supernatants containing 90% methanol were also acidified with TFA and then dried in a Speedvac apparatus (Savant Instruments, Farmingdale, NY, USA). Residues were redissolved in water/acetonitrile/TFA (80:20:0.1), and aliquots were taken for liquid scintillation counting prior to analysis by HPLC. All samples were analyzed on a Delta Pak C_{18}-300 Å (3.9 mm × 15 cm, 5 µm) column using a 50-min gradient (3–53 min) of 20–30% (vol.) acetonitrile in water with 0.1% TFA in each solvent, followed by a 10 min rinse with 60% acetonitrile. Effluents were monitored at 214 nm, and fractions

were collected, Speedvac-dried, redissolved in 500 µl of water/acetonitrile/TFA (80:20:0.1 by vol.), mixed with 4 ml Ecoscint A (National Diagnostics, Manville, NJ, USA), and counted in a Tri-Carb Model 460C liquid scintillation system (Packard, Downers Grove, IL, USA)[20]. Tritium recoveries were about 90% both before and after HPLC analyses, and fractions corresponding to the 60% acetonitrile rinse did not show significant radioactivity.

The hemolymph contained some UV-absorbing components that were neither eliminated by the initial treatments (gel filtration, dialysis) nor by various protein precipitation methods at the end of the incubations (using methanol, TFA, or acetonitrile-TFA mixtures). As a result of the incomplete purification, hemolymph control samples without added [^{3}H]NLPBAN showed a number of A_{214} peaks that not only interferred with an exact quantification of NLPBAN but possibly also obscured NLPBAN hydrolysis products. Thus, although NLPBAN peaks were generally smaller after incubation than before, no new A_{214} peaks were detected. Quantitative analysis of the incubation mixtures, therefore, rested on the tritium counts in the HPLC fractions. Whereas this approach adequately measured the amount of NLPBAN converted during the incubation, it could not give a complete picture of the hydrolysis products, because the parent [^{3}H]NLPBAN molecule was labeled in only two positions, Tyr^{15} and Tyr^{28}, and any fragment not including one of these residues would be undetectable. The [^{3}H]NLPBAN counts recovered after hemolymph incubations were converted to percent of total dpm and subtracted from the corresponding percentages of buffer controls incubated for the same time, to obtain the net NLPBAN loss attributable to peptidase action and to compute the reaction rates.

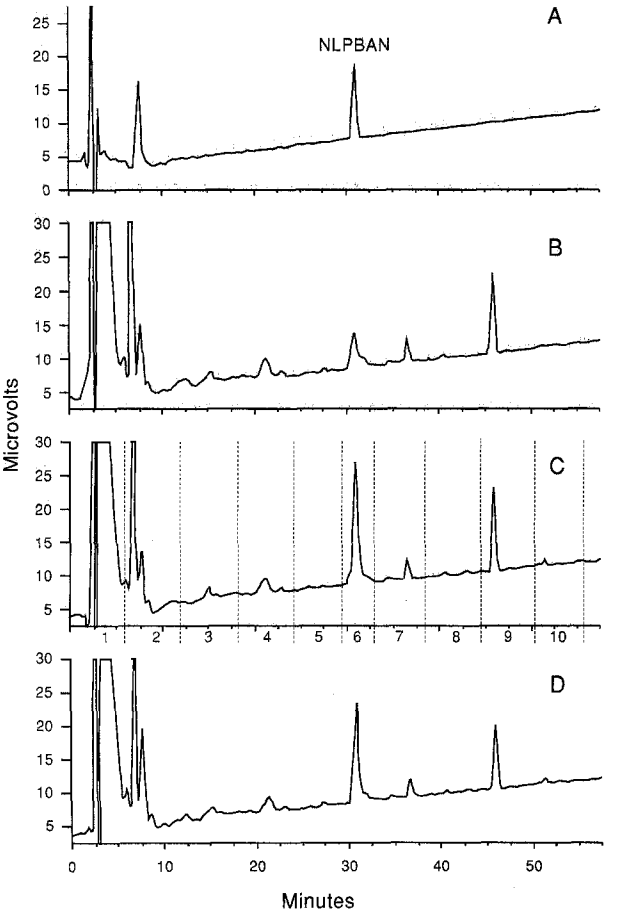

Figure 1. HPLC of incubation mixtures. *A* [^{3}H]NLPBAN (0.52 µg) and buffer (total vol. 150 µl), incubated for 4 h at 30 °C; *B* 38 µl dialyzed *M. sexta* hemolymph (2.0 mg protein) in buffer, incubated for 4 h at 30 °C; *C* [^{3}H]NLPBAN and 38 µl dialyzed hemolymph in buffer, 0 h control; *D* [^{3}H]NLPBAN and 38 µl dialyzed hemolymph in buffer, incubated for 4 h at 30 °C. Methanol (9 vol.) was added to each sample, and they were processed as described in 'Materials and methods'. An aliquot corresponding to 55% of the incubation mixture was used for each analysis. Ordinate shows A_{214} in integrator units (µV), 1 V being equal to 1 absorbancy unit. Fractions used for other experiments are shown in panel *C*. HPLC segment corresponding to 60% acetonitrile rinse is not shown.

Results and discussion

Figure 1 shows examples of the HPLC analyses of various incubation mixtures. Dialyzed *M. sexta* hemolymph incubated without [^{3}H]NLPBAN (fig. 1B) showed a number of A_{214} peaks, one of them coinciding with and somewhat broader than the NLPBAN peak in the [^{3}H]NLPBAN-buffer control incubation (fig. 1A). Accordingly, the [^{3}H]NLPBAN-hemolymph 0 h control mixture (fig. 1C) showed a larger NLPBAN peak than either [^{3}H]NLPBAN or hemolymph alone. After 4 h of incubation the NLPBAN peak in the [^{3}H]NLPBAN-hemolymph mixture was slightly reduced in size (fig. 1D). The distribution of radioactivity in HPLC fractions obtained from a similar experiment is shown in figure 2. A 4 h incubation in buffer caused a very small decrease in amount of [^{3}H]NLPBAN recovered (fraction 6) compared to the 0 h control. The 4 h incubation with hemolymph caused a moderate decrease in the amount of [^{3}H]NLPBAN (11% or 13% compared to 4-h buffer or 0-h hemolymph control, respectively), accompanied by increases of the ^{3}H label in fractions 1–3 to more than twice the percentage found in controls. These results suggest that several tritium-labeled [^{3}H]NLPBAN fragments were formed by *M. sexta* hemolymph peptidase(s). However, as might be expected from the small decrease in UV absorption of the NLPBAN peak, the NLPBAN degradation did not give rise to any UV-measurable products in the areas of fractions 1–3 (fig. 1C, D). (A peak at 7.5 min originated from the buffer.) The ^{3}H label in fraction 7 also decreased as a result of the incubation with hemolymph. Apparently, a labeled compound or compounds eluting after [^{3}H]NLPBAN were also converted by hemolymph enzyme(s).

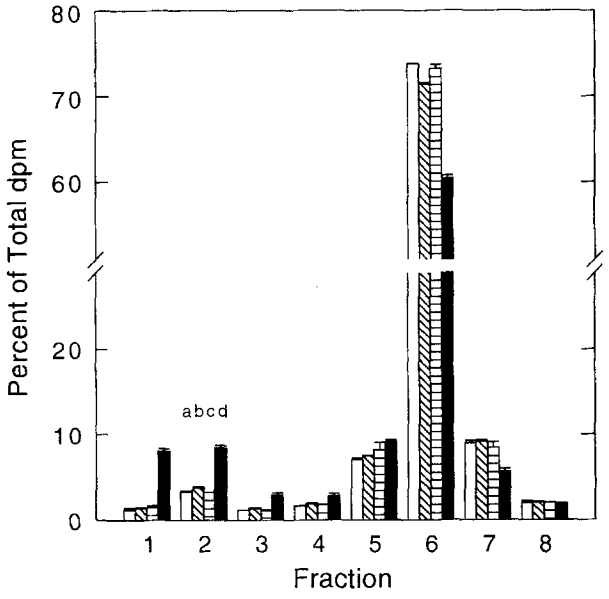

Figure 2. Distribution of ^3H radioactivity in HPLC fractions (see fig. 1C) of incubation and control mixtures. The four columns for each fraction (left to right) show the results for *a* [^{3}H]NLPBAN (9,000 dpm; 0.52 µg) and buffer (total vol. 150 µl), 0 h control; *b* [^{3}H]NLPBAN and buffer, incubated for 4 h at 30 °C; *c* [^{3}H]NLPBAN and 19 µl Sephadex G-25-filtered *M. sexta* hemolymph (0.8 mg protein) in buffer, 0 h control; *d* same mixture as in *c*, but incubated for 4 h at 30 °C. Ordinate shows recovery in individual fractions as percent of total dpm recovered from HPLC. Fractions 9 and 10 did not contain significant radioactivity. Error bars indicate SD of duplicate assays.

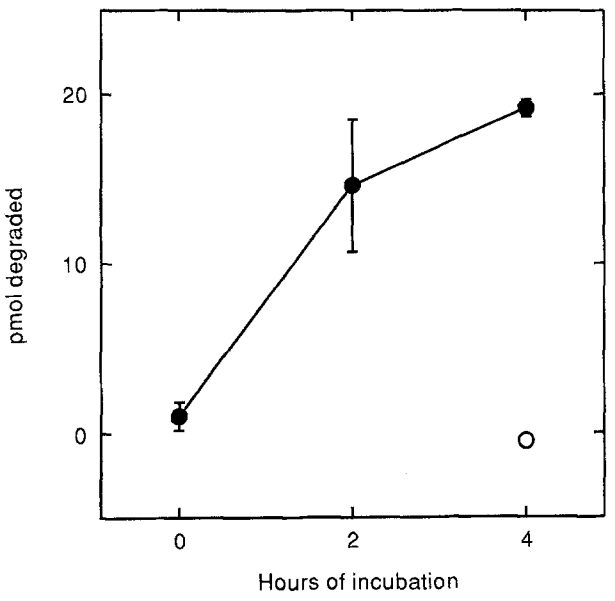

Figure 3. Degradation of [^{3}H]NLPBAN. [^{3}H]NLPBAN (0.52 µg = 133 pmol) and 19 µl Sephadex G-25-filtered *M. sexta* hemolymph (0.8 mg protein) were incubated in buffer (total vol. 150 µl) at 30 °C for the indicated times (●). (○), boiled hemolymph control. The amounts of [^{3}H]NLPBAN degraded were calculated from the differences in [^{3}H]NLPBAN recoveries between incubations with hemolymph and buffer controls (see 'Materials and methods'). Means ± SD of duplicate assays.

As shown in figure 3 the rate of [^{3}H]NLPBAN hydrolysis was very low, 6.4 fmol/min/µl hemolymph equivalent during the first 2-h interval and decreasing thereafter. Other experiments yielded rates between 2 and 10 fmol/min/µl. Dialysis did not significantly affect the enzyme activities. Incubations with undialyzed or dialyzed *M. sexta* hemolymph samples gave almost identical conversion rates. Boiled hemolymph did not hydrolyze [^{3}H]NLPBAN (fig. 3). Incubations of two samples of undialyzed *H. zea* hemolymph with [^{3}H]NLPBAN yielded rates of 1.6 and 2.4 fmol/min/µl and the same HPLC radioprofile as that obtained with *M. sexta* hemolymph. Optimal pheromone synthesis in *H. zea* is obtained by injection of as little as 4 pmol/female[2]. In the 50 µl hemolymph volume of a female moth[11] this would result in a concentration of 80 nM. The NLPBAN concentration used in the current study was 0.9 µM, i.e. about 10 times the hemolymph concentration required for optimal pheromonotropic response. Thus, the in vivo rates of NLPBAN degradation may be lower than the 1.6–2.4 fmol/min/µl measured in vitro (equivalent to 80–120 fmol/min/female). Following injection of 100 pmol (equivalent to 2.0 µM in 50 µl hemolymph) Hez-PBAN into ligated *H. zea* females, marginally effective levels of the peptide persisted in the hemolymph for 1–2 hours[19]. This would indicate an inactivation rate comparable to the rates obtained in vitro at a comparable initial PBAN concentration.

Previously reported neuropeptide hydrolysis rates in hemolymph were much higher than those obtained in the present study but were obtained with 100–250 times higher substrate concentrations. With a proctolin concentration of 0.25 mM, *P. americana* hemolymph yielded rates in the pmol/min/µl range (deduced from fig. 1)[13]. *Manduca* AKH (at 0.1 mM) was hydrolyzed by *M. sexta* larval hemolymph at a rate of 0.65 µg/h/50 µl or about 0.2 pmol/min/µl[15]. The AKH-hydrolyzing endopeptidase was apparently not saturated at an AKH concentration of 10 µM and was inactive at ≤0.1 µM, leaving its physiological function somewhat in question[15]. Having focused on the metabolism of [^{3}H]NLPBAN at a very low substrate concentration, we do not yet know how this concentration relates to the K_M or substrate saturation level of the NLPBAN-hydrolyzing enzyme(s).

Various peptidase inhibitors were tested for their effects on [^{3}H]NLPBAN hydrolysis by *M. sexta* hemolymph peptidases. With added ethylenediaminetetraacetic acid (EDTA) or 1,10-phenanthroline (both at 1.0 mM), the [^{3}H]NLPBAN recoveries were no different from those of the incubation mixtures without inhibitors (fig. 4). Thus, the initial step in the [^{3}H]NLPBAN degradation was not inhibited by either one of these inhibitors. However, both inhibitors decreased the ^{3}H yields in HPLC fraction 1 and marginally in fraction 2. The effects of leupeptin (20 µM), amastatin (0.1 mM),

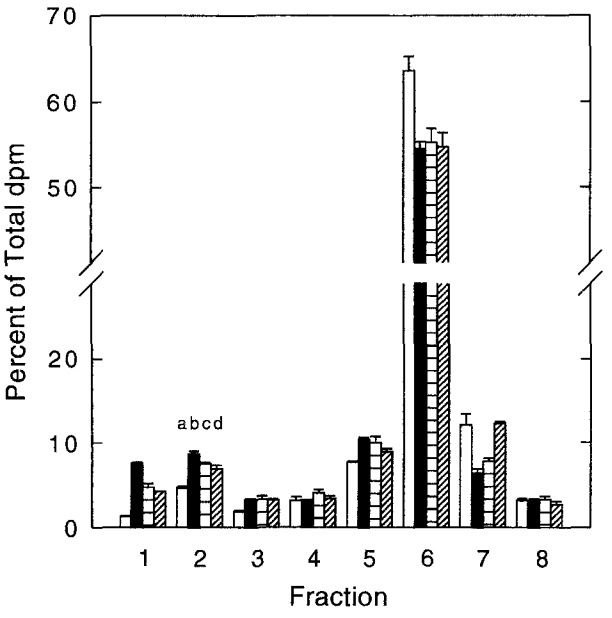

Figure 4. Radio-HPLC analyses of the effects of inhibitors on [^{3}H]NLPBAN degradation. The four columns for each fraction (left to right) show the results for *a* [^{3}H]NLPBAN (9,000 dpm; 0.52 µg) and buffer (total vol. 150 µl), incubated for 4 h at 30 °C; *b* [^{3}H]NLPBAN and 38 µl dialyzed *M. sexta* hemolymph (2.0 mg protein) in buffer, incubated for 4 h at 30 °C; *c* same mixture as in *b*, but with addition of 1.0 mM EDTA; *d* same mixture as in *b*, but with addition of 1.0 mM 1,10-phenanthroline. Otherwise as figure 2.

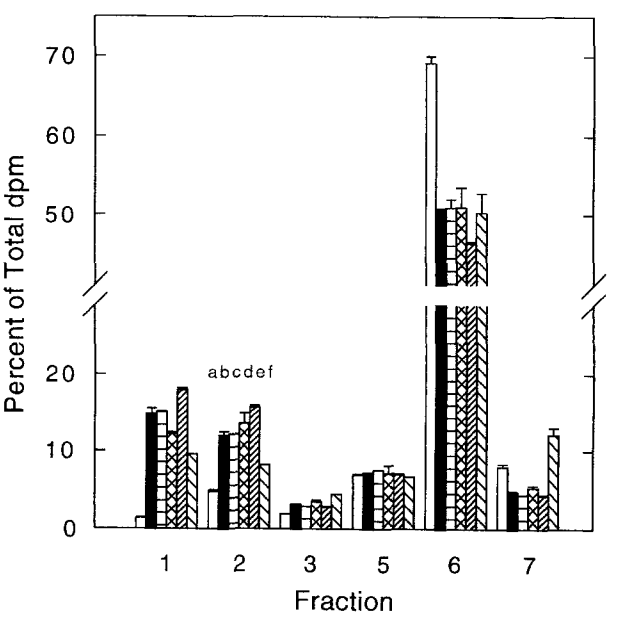

Figure 5. Radio-HPLC analyses of the effects of inhibitors on [^{3}H]NLPBAN degradation. The six columns for each fraction (left to right) show the results for *a* [^{3}H]NLPBAN (9,000 dpm; 0.52 µg) and buffer (total vol. 150 µl), incubated for 4 h at 30 °C; *b* [^{3}H]NLPBAN and 38 µl dialyzed *M. sexta* hemolymph (1.6 mg protein) in buffer, incubated for 4 h at 30 °C; *c* same mixture as in *b*, but with addition of 20 µM leupeptin; *d* same mixture as in *b*, but with addition of 0.1 mM amastatin; *e* same mixture as in *b*, but with addition of 1.0 mM EGTA; *f* same mixture as in *b*, but with addition of 2.0 mM AEBSF. Fractions 4 and 8 did not show differences between the samples and were omitted. Otherwise as figure 2.

ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 1.0 mM), and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF; 2.0 mM) were tested in another experiment (fig. 5). None of these compounds inhibited the initial step in the [^{3}H]NLPBAN degradation. EGTA slightly increased the rate of [^{3}H]NLPBAN conversion and increased the ^{3}H yields in fractions 1 and 2. AEBSF increased the ^{3}H yield in fraction 7 and decreased the ^{3}H yields in fractions 1 and 2. In summary, none of the inhibitors had a significant effect on the initial NLPBAN conversion in *M. sexta* hemolymph, but some of them changed the radio-HPLC profile of the hydrolysis products, possibly by affecting peptidases involved in the secondary degradation of the initial metabolites. The enzyme(s) responsible for the primary attack on NLPBAN seem(s) to be remarkably resistant to a variety of peptidase inhibitors, including those specific for serine proteases (AEBSF) and metalloproteases (EDTA, EGTA, 1,10-phenanthroline), and thus do(es) not appear to be (a) typical serine or metallopeptidase(s). That clearly distinguishes it (them) from the *M. sexta* (metallo)endopeptidase hydrolyzing AKH[15]. Leupeptin, an endopeptidase inhibitor, and amastatin, an aminopeptidase inhibitor, also failed to inhibit NLPBAN degradation. Based on current information the NLPBAN-degrading enzyme(s) cannot be clearly categorized. Our data do not positively identify it (them) as peptidase(s), but given the peptide nature of NLPBAN and the demonstrated presence of peptidases in hemolymph of other insect species[12–14] and in larvae of *M. sexta*[12,15], it is reasonable to assume that the NLPBAN-degrading enzyme(s) in *M. sexta* adults is (are) peptidase(s) as well. The rate of the observed NLPBAN degradation is in a plausible range and could be physiologically relevant.

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