Neuropeptides of the silkworm, *Bombyx mori*

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Abstract. Six neuropeptides of the silkworm, *Bombyx mori*, have been isolated and chemically characterized during the past 10 years. They are bombyxin, prothoracicotropic hormone, pheromone-biosynthesis-activating neuropeptide/melanization-and-reddish-coloration hormone, diapause hormone, eclosion hormone, and adipokinetic hormone. Recent progress in research on these neuropeptides is described.
Key words. Insect neuropeptide; silkworm; *Bombyx mori*; amino acid sequence; immunohistochemistry.

Insect neuropeptides are involved in various processes such as molting, metamorphosis, reproduction and metabolism. The silkworm, *Bombyx mori*, has been reared in sericulture for several thousand years, and now it may be the only insect that is collected on the scale of millions of individuals. Many investigators have considered the silkworm as a representative of insects from various standpoints, and accumulated a vast amount of data on genetics, physiology, etc. The largest number of neuropeptides have been isolated and characterized from the silkworm, *B. mori*. Figure 1 shows the amino acid sequences of *Bombyx* neuropeptides which have been characterized thus far. In the following sections, recent progress in the research on these neuropeptides is described.

Bombyxin

Bombyxin can induce adult development in a brainless pupa if the saturniid moth, *Samia cynthia ricini*[5]. It also stimulates *Samia* prothoracic glands cultured in vitro to produce ecdysone[28]. However, it has no ability to induce adult development in a brainless pupa of *B. mori*[5], although it was isolated from *Bombyx* heads. Its intrinsic function in *B. mori* is still unknown.

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Bombyxin                        *         *
    A chain  -II  GIVDECCLRPCSVDVLLSYC
             -IV  -V-----IQ--TL---AT--

    B chain  -II <QQPQAVHTYCGRHLARTLADLCWEAGVD
             -IV  -EANVA-H-------N-------DTS-E

                 S-S: A6-A11, A7-B10, A20-B22.

Prothoracicotropic hormone (PTTH)
            *         *         *         *#        *         *
    GNIQVENQAIPDPPCTCKYKKEIEDLGENSVPRFIETRNCNKTQQPTCRPPYICKESLYS
            *         *         *         *
    ITILKRRETKSQESLEIPNELKYRWVAESHPVSVACLCTRDYQLRYNNN

      #: carbohydrate moiety linked.

Eclosion hormone (EH)
            *         *         *         *         *         *
    SPAIASSYDAMEICIENCAQCKKMFGPWFEGSLCAESCIKARGKDIPECESFASISPFLNKL

    S-S: 14-38, 18-34, 21-49.
    EH-I, 3-61; EH-II, 1-61; EH-III, 3-62; EH-IV, 1-62.

Pheromone biosynthesis activating neuropeptide (PBAN)/Melanization
  and reddish coloration hormone (MRCH)
                                *         *         *
    PBAN-I/MRCH-I  LSEDMPATPADQEMYQPDPEEMESRTRYFSPRL(amide)
    PBAN-II        R--------------------------------(amide)

Adipokinetic hormone (AKH)

    <QLTFTSSWG(amide)

Diapause hormone (DH)
            *         *
    TDMKDESDRGAHSERGALCFGPRL(amide)
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Figure 1. Amino acid sequences of *Bombyx* neuropeptides.

Five molecular species of bombyxin have been isolated and characterized. They differ from one another by the substitution of some amino acid residues. Up to now, the amino acid sequences of two out of five molecular species, bombyxin-II[30,31] and -IV[20], have been determined completely (fig. 1). Bombyxin consists of two non-identical peptide chains, A and B chains, which are held together by disulfide bonds. A and B chains comprise 20 and 28 amino acid residues, respectively. Detailed analysis of thermolysin digests of bombyxin-II established the arrangement of three disulfide bonds between Cys^{A6} and Cys^{A11}, Cys^{A7} and Cys^{B10}, and Cys^{A20} and Cys^{B22}. Bombyxins have about 40% sequence homology with vertebrate insulins[29,30], and on the basis of this sequence homology the tertiary structure of bombyxin-II was estimated to be similar to that of insulins by computer graphics and energy minimization techniques[9]. The results showed that the residues which contribute to forming a hydrophobic core in the center of the molecule are especially highly conserved, while the surface structures are considerably different from each other. This might account for the fact that insulin has no activity in the *Samia* assay system and that anti-insulin antibody does not recognize bombyxins[29].

When the structure of bombyxin-II was determined completely, studies on chemical synthesis of bombyxins were started, focusing mainly on the formation of the three disulfide bonds. At the first stage of the synthetic study, A and B chains were allowed to bind by non-selective formation of disulfide bonds[31]. A and B chains with thiol groups at all Cys residues were prepared first by solid-phase peptide synthesis followed by deprotection and cleavage from the resins. Then they were put together and subjected to air-oxidation in a high pH buffer solution. Bombyxin-II, both structurally and biologically identical with the natural bombyxin-II, was obtained, but the yield was only 4%. Subsequently, many efforts were made to develop a method for more selective formation of the disulfide bonds to give bombyxins in a

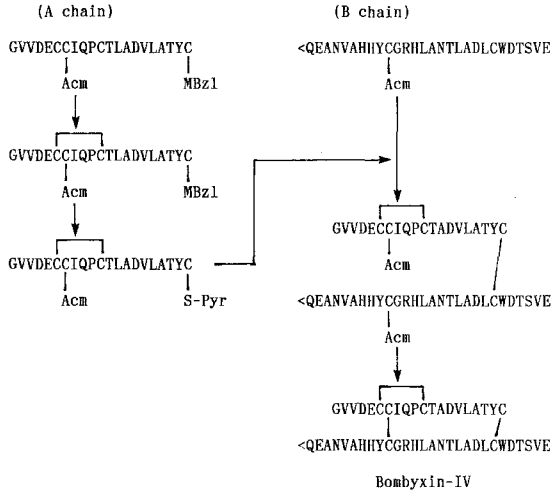

Figure 2. Synthetic scheme for bombyxin-IV. Acm: acetamidomethyl; MBzl: methoxybenzyl; S-Pyr: S-pyridil; <Q: pyroglutamyl.

higher yield. The two chains were coupled semi-selectively by 2-step formation of disulfide bonds[21]. First, [Cys(Acm)^{6,7,11}] A chain (Acm: acetamidomethyl) and [Cys(Acm)^{10}]B chain were prepared. Then, by coupling these peptides [Cys(Acm)^{A6,A7,A11,B10}]bombyxin-IV was synthesized nearly quantitatively, which afforded bombyxin-IV by iodine oxidation in 8% yield. Recently, bombyxin-II and -IV have been synthesized by stepwise, regioselective formation of the three disulfide bonds in a yield of 50–60%[22]. Figure 2 shows the scheme for this synthesis. Three kinds of protecting groups for sulfhydryl groups, which could be deprotected selectively, were used. This method may be applicable to the synthesis of other insulin-like molecules containing three disulfide bonds.

A monoclonal antibody was produced against an amino-terminal decapeptide of the bombyxin-I A chain[26]. This antibody did not recognize the whole molecule of bombyxins, but only A chains generated by reduction of bombyxins with dithiothreitol or mercaptoethanol. Native PAGE analyses by immunoblotting using this antibody demonstrated that bombyxin consists of many molecular species, as had been expected from purification studies. Immunohistochemistry using this antibody indicated that bombyxin was produced in four pairs of mid-dorsal neurosecretory cells and transferred to the corpora allata[26]. The bombyxin content in the brain estimated by immunohistochemistry, immunoblotting and bioassay changed remarkably during larval-larval and larval-pupal development[27].

A genomic DNA encoding bombyxin was isolated by screening a genomic library with two oligonucleotide probes synthesized on the basis of the amino acid sequence[8]. The nucleotide sequence of this DNA showed that it encodes preprobombyxin (signal peptide/B chain/two basic amino acid residues/C peptide/two basic amino acid residues/A chain) and that it has considerable homology with insulin genes, as would be expected from the homology in the amino acid sequence. The overall organization of the preprobombyxin gene is the same as that or preproinsulin genes. These facts suggest that bombyxin is biosynthesized in the same manner as are insulins; preprobombyxin is first translated, the signal peptide is then cut off, three disulfide bonds are formed, and finally the C-peptide is cleaved off to generate a mature bombyxin molecule. The bombyxin gene has no intron, in contrast to other members of the insulin-gene family.

In another study, two independent cDNA clones were isolated by screening a cDNA library prepared from 16,000 brains with an oligonucleotide probe[1]. One had the complete coding region and the other did not. Domain organization was naturally the same as in the genomic DNA. Genomic Southern hybridization analysis using this cDNA as a probe demonstrated that the *Bombyx* genome contains multiple copies of the bombyxin gene. Some of them were cloned and sequenced. They were classified into two families, A and B, according to the sequence similarity[11]. In a typical case two genes, each belonging to a different family, are closely apposed to form a pair with opposite orientation, and most of the bombyxin gene copies are clustered in at least three regions. Later, C-family genes were cloned[7]. The existence of multiple bombyxin gene copies in one *Bombyx* genome contrasts strongly with vertebrate insulin genes, which exist in a single copy or two copies per haploid genome. Northern hybridization experiments demonstrated that only brain, of the tissues so far examined, contained a transcript. The localization of the bombyxin mRNA examined by in situ hybridization indicated that the transcripts are produced in four pairs of mid-dorsal neurosecretory cells[6], which are also immunoreactive to a monoclonal antibody as described above.

Prothoracicotropic hormone (PTTH)

Bombyx PTTH can induce adult development in a *Bombyx* brainless pupa but not in a *Samia* brainless pupa[5]. Recently, PTTH has been characterized as a homodimer of a 109-amino acid residue peptide[10,12]. Peptide sequencing of the purified PTTH and its enzymatic fragments revealed that the PTTH monomer consisted of at least 104 amino acid residues, though the residue at position 41 could not be identified and there was microheterogeneity in the amino-terminal section resulting from truncation of the amino-terminal 6 and 7 residues[10]. On the other hand, cDNA for *Bombyx* PTTH has been cloned and its nucleotide sequence determined[12]. The amino acid sequence deduced from the nucleotide sequence suggested that *Bombyx* PTTH monomer consists of 109 amino acid residues. The sequence of 104 amino acid residues determined by peptide sequencing was completely identical with the sequence deduced from the amino-terminus. The carboxyl-terminal five residues, -R-Y-N-N-N, could not be identified by

peptide sequencing. This was perhaps due partly to the extremely minute amount of peptides available, and partly to the possible truncation of several amino acid residues at the carboxyl-terminus. The residue at position 41 was deduced to be asparagine. Since a threonine residue exists at position 43, it is most likely that a carbohydrate moiety is linked to the side chain of this asparagine, because the sequence, -N-X-T-, is generally recognized as a consensus sequence for asparagine N-glycosylation. Thus *Bombyx* PTTH may be a glycoprotein. The structure of the carbohydrate moiety remains unclear.

SDS polyacrylamide gel electrophoresis of the purified PTTH revealed that its molecular weight is about 30,000. After reduction of the PTTH with dithiothreitol, the band completely disappeared and two close bands corresponding to the molecular weight of 16,000–17,000 newly appeared, suggesting that the PTTH consists of two essentially identical glycopeptides held together by disulfide bond(s)[10].

A monoclonal antibody was produced against a synthetic pentadecapeptide corresponding to the amino-terminal portion of the PTTH monomer[27]. Immunohistochemistry of the *Bombyx* brain-corpora cardiaca-corpora allata complex using this antibody indicated that the PTTH is produced in two pairs of dorso-lateral neurosecretory cells in the brain and transferred to the corpora allata. This antibody was also used for isolating a PTTH cDNA.

The nucleotide sequence of the PTTH cDNA suggested that it encodes prepro-PTTH[12]. The cDNA contains an open reading frame that encodes 224 amino acid residues consisting of a putative signal peptide (29 amino acid residues), two unknown peptides (21 and 57 amino acid residues) and a PTTH monomer in this order from the 5′ end. Since these three peptides are separated by two or three basic amino acid residues, they may be cut off from one another by proteolytic processing after translation. In contrast to bombyxin, the nucleotide and deduced amino acid sequences of prepro-PTTH did not show homology with other known proteins. In situ hybridization with a ^{35}S-labeled complementary RNA probe revealed that the PTTH gene transcript was localized in the same cells as those that were stained immunohistochemically, as described above. When a portion of the cDNA encoding the PTTH monomer was inserted into a plasmid vector and introduced into *Escherichia coli*, it produced PTTH as a dimer without a carbohydrate moiety. This synthetic PTTH was slightly less potent than the natural one, suggesting that the carbohydrate moiety was not essential to the activity. The PTTH monomer has six Cys residues. Determination of the arrangement of the disulfide bonds is now in progress.

Eclosion hormone (EH)

EH induces a series of eclosion phenomena as well as larval-larval and larval-pupal ecdysis. EH was extracted from pharate adult heads and purified to homogeneity. Four molecular species of EH were isolated[16,17] and named EH-I–-IV in the order of elution from reverse phase HPLC. The yields of EH-I–-IV from 770,000 heads were 16.0, 5.5, 7.5 and 2.8 µg, respectively. The elution positions of EH-I and -II were very close, and so were those of EH-III and -IV, but the former two and the latter two eluted at positions rather distant from each other. An aliquot of each EH was subjected to automated Edman degradation, revealing that these four EHs had the same sequence from the amino-terminus to the 25th residue except for truncation of the two amino-terminal residues, Ser-Pro, in EH-I and -III. The amount of each EH isolated was so small that the whole sequence of EHs was constructed by combining the sequence data from each EH. Sequence analyses of fragments generated by lysyl endopeptidase digestion of EH-III and of those by V8 protease digestion of EH-II established a single sequence consisting of 61 residues from the amino-terminus[16]. No more sequence data were obtained from sequence analyses of natural EHs. The exact sequences of EH-I–-IV were established by using EH synthesized in *Escherichia coli* as described later.

An EH gene was cloned and its nucleotide sequence indicated that the deduced amino acid sequence of EH has 62 residues[32], longer than the sequence determined by the peptide sequencing by one residue, which corresponds to the carboxyl-terminus, Leu. Then a gene encoding EH(1-62) was chemically synthesized taking account of the codon usage in *E. coli*. The gene was inserted into a secretion vector and expressed in *E. coli*, and led to the production of biologically active EH[18]. Digestion of this synthetic EH with carboxypeptidase A for only 1 min gave EH(1-61) nearly quantitatively. The elution positions of the synthetic EHs, EH(1-61) and EH(1-62), and the natural EHs, EH-I–-IV, on reverse phase HPLC were compared: EH-I and -II were eluted at a position almost identical with that of EH(1-61), and EH-III and -IV nearly overlapped with EH(1-62). These facts indicate the EH-I and -II terminate at the 61st residue and EH-III and -IV at the 62nd residue. That EH-I and -II terminate at the 61st residue was proved by the fact that their carboxyl-terminal fragment, obtained after V8 protease digestion, coeluted with synthetic EH(51-61) but not with EH(51-62). Thus, it was concluded that EH-I–-IV have the sequences 3-61, 1-61, 3-62 and 1-62, respectively[17].

In order to know whether the truncation of the carboxyl-terminal Leu in EH-I and -II was artefactual or not, the synthetic EH(1-62) was incubated with crude saline extract of *Bombyx* pharate adult heads on ice for 3 h. The results indicated that most EH(1-62) were converted to EH(1-61). This conversion was completely surpressed by changing the extraction solvent from 2% NaCl to 1 M acetic acid/20 mM HCl. These facts suggest that EH-I and -II were produced artefactually from EH-III and -IV, respectively, during extraction, possibly by digestion

with carboxypeptidase A-like enzyme in the extract[17]. It is not clear whether the amino-terminal truncation of two residues in EH-I and -III occurred artefactually or not.

EH has six Cys residues. These may form three pairs of disulfide bonds, because EH was inactivated by reduction with dithiothreitol. The arrangement of the disulfide bonds was estimated by using the synthetic EH(1-62)[18]. Thermolysin digestion of the synthetic EH generated 11 cystine-containing peptides, all of which were sequenced. The results clearly indicated that there are three disulfide bonds between Cys^{14} and Cys^{38}, Cys^{18} and Cys^{34}, and Cys^{21} and Cys^{49}. Since the specific activity of this synthetic EH was approximately the same as that of natural EH, it is most likely that natural EH has disulfide bridges at the same positions.

A monoclonal antibody was produced using as antigen a synthetic fragment corresponding to the carboxyl-terminal portion, EH(49-61), coupled with bovine serum albumin[15]. This antibody recognized both the synthetic fragment and natural EHs as examined by competitive ELISA. Immunohistochemistry using this antibody indicated that EH is produced in two pairs of median neurosecretory cells of the brain. The four cells are supposed to be located in the anterior portion of the brain as observed by whole mount staining. This antibody could also be applied to affinity chromatography, in which the antibody was linked to CNBr-activated Sepharose 4B. When the partially purified preparation of EH was applied to the affinity column, most of the materials were passed through the column and EH activity was recovered by elution of the synthetic fragment. EH was purified to homogeneity by one more step of purification by reverse phase HPLC. Therefore, if this affinity chromatography is adopted in the purification of EH from *Bombyx* heads, the purification scheme would become much simpler, because several steps of HPLC purification can be omitted.

Pheromone biosynthesis activating neuropeptide (PBAN)/melanization and reddish coloration hormone (MRCH)

PBAN is possibly produced in the subesophageal ganglion of female moths, is secreted into the hemolymph, and stimulates the pheromone gland to synthesize sex pheromone(s). PBAN has been isolated and characterized from two species of moths, *Heliothis zea* and *Bombyx mori*. Two molecular forms of *Bombyx* PBAN, PBAN-I[14] and -II[13], have been characterized as single-chain linear peptides consisting of 33 and 34 amino acid residues, respectively. PBAN-II has the same sequence as PBAN-I except for an additional Arg residue at the amino-terminus of PBAN-I. PBAN-I has 82% sequence homology with the *Heliothis* PBAN.

MRCH is associated with color polymorphism. It was isolated from the same source using an armyworm cuticle melanization assay, though its function in relation to the color change in *B. mori* is still unknown. Three molecular forms of MRCH, MRCH-I, -II and -II, were isolated[23]. Only MRCH-I has been completely sequenced, demonstrating that MRCH-I is the same molecule as PBAN-I[24]. Natural and synthetic PBAN-I (MRCH-I) revealed interspecific activity both in cuticular melanization and in sex pheromone production.

The structure-activity relationship of PBAN-I has been extensively studied[19]. PBAN-I has three Met residues at positions 5, 14 and 22. Oxidation of all the Met residues to Met(0), methionine sulfoxide, resulted in a dramatic increase in activity. However, it is unclear which Met(0) contributes most to increasing the activity. The carboxyl-terminal amide is indispensable for the activity. During sequence analysis of PBAN-I, it was found that the carboxyl-terminal decapeptide generated after enzymatic digestion had a rather low but definite activity. Thereafter many peptide analogs related to this carboxyl-terminal peptide were synthesized, and their activities were assessed. The smallest peptide with PBAN activity was a carboxyl-terminal pentapeptide. Similar results were also obtained in *Heliothis* PBAN. Modification of the amino-terminal amino group of the carboxyl-terminal hexapeptide and pentapeptide of PBAN-I by acetylation, benzoylation etc. resulted in marked increase in activity, possibly due to the peptides becoming much more tolerant to proteolytic enzymes, especially aminopeptidase, in the hemolymph.

To understand the mode of action of PBAN, ^{14}C-hexadecanoic acid was applied topically to the pheromone gland of a decapitated virgin female moth which has been injected with PBAN[2]. The ^{14}C-incorporation into bombykol and C_{16}-fatty alcohol increased, whereas that into the unsaturatd C_{16}-fatty acyl moiety did not, suggesting that the main regulatory role of PBAN is in the reduction of the fatty acyl moiety rather than in its desaturation. The fact that the pheromone gland from a decapitated moth cultured in vitro in the presence of PBAN produced sex pheromone clearly indicated that PBAN directly stimulates the pheromone gland[2].

Adipokinetic hormone (AKH)

AKH acts on the fat body, causing diglyceride release into the hemolymph. It is the first peptide to have been characterized in insects, as early as 1976. The amino acid sequence of AKH was determined first from the locusts, *Schistocerca gregaria* and *Locusta migratoria*, then from the tobacco hornworm, *Manduca sexta*, and the corn earworm, *Heliothis zea*, and quite recently from the silkworm, *B. mori*[4]. *Bombyx* AKH was isolated from adult heads by a 9-step purification procedure. The amino acid sequence was determined by FAB-MS measurement of the intact peptide and sequence analysis of the deblocked peptide obtained after pyroglutaminate aminopeptidase digestion. The determined sequence of *Bombyx* AKH

was identical with that of *Manduca* and *Heliothis* AKHs. In *Bombyx*, this AKH elevated lipid level in the hemolymph in a dose-dependent manner, but did not affect carbohydrate level.

Diapause hormone (DH)

Diapause occurs at the embryonic stage in *B. mori*. DH is known to be produced in the female subesophageal ganglion and to induce embryonic diapause during the pupal stage. DH was extracted from 55,000 complexes of the subesophageal ganglion and the first thoracic ganglion of day 1 pupae with hot water. The extract was subjected to two steps of reverse phase HPLC. The second HPLC effected the isolation of DH (less than 0.5 μg) and the pure DH induced diapause in 20% eggs at a dose of 1.5 ng/pupa [3].

The amino acid sequence was analyzed using 0.15 μg of the isolated DH. The results indicated a sequence consisting of 24 amino acid residues (fig. 1). The sequence from Arg^{15} to Gly^{21} was confirmed by sequencing one major fragment generated by endoproteinase Glu-C digestion of DH. In order to determine the carboxyl-terminal structure, two peptides with the same amino acid sequence were synthesized; one was amidated and the other free acid at the carboxyl-terminus. The synthetic peptide with free acid had no activity even when as much as 1 μg was injected into a pupa. By contrast, the amidated peptide showed activity comparable to the natural DH. Besides, it was eluted at the same retention time as the natural peptide on reverse phase HPLC, establishing the structure as an amidated peptide with 24 amino acid residues.

DH has a sequence homology with PBAN/MRCH and myotropic peptides in the several carboxy-terminal residues. It is of interest that the homologous part is confined to the carboxyl-terminal section including an amide structure, the most important part for displaying activities of PBAN/MRCH and myotropic peptides, although there is no homology in other parts of the sequence.

Conclusion

Major neuropeptides in *B. mori* have been isolated and sequenced completely. No sequence homology with vertebrate peptides has been found in PTTH, EH, AKH or DH, all of which are related to molting, metamorphosis or diapause, characteristic of insect development. Some of the peptides are homologous with the peptides of other insects or crustaceans: DH with PBAN/MRCH in the carboxyl-terminal part, and with myotropic peptides of cockroach and locust; and AKH with the red-pigment-concentrating hormone (RPCH) of shrimp. By contrast, bombyxin and PBAN/MRCH have some homology with vertebrate insulin-family peptides, though the homology of the latter does not seem so significant. It is of interest to consider how these peptides have evolved, and whether peptides homologous with PTTH, EH or DH might be present in animals other than insects.

All the *Bombyx* neuropeptides mentioned above are now available in quantity, because smaller peptides (bombyxin, PBAN/MRCH, AKH and DH) have been synthesized chemically, and larger peptides (PTTH and EH) produced by recombinant DNA techniques in *E. coli*. Thus we are in a new stage, in which the pure synthetic neuropeptides can be used to see direct effects on a whole organism or an organ cultured in vitro. Furthermore, the availability of such synthetic peptides and their analogs will permit us to study their mode of action, for example the interaction between neuropeptides and their putative receptors, and signal transduction. It will also enable us to gain more precise knowledge about the peptide molecules themselves, for example a better understanding of their secondary and tertiary structures, which may be important in considering how they interact with their receptors.

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Adipokinetic hormones: Cell and molecular biology

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Abstract. Adipokinetic hormones AKH I (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH_2) and AKH II (pGlu-Leu-Asn-Phe-Ser-Trp-Gly-Thr-NH_2) are synthesized by neurosecretory cells (NSC) of the corpora cardiaca (CC) in the locust, *Schistocerca gregaria*. These NSC constitute a homogeneous 'peptide factory' as each cell synthesizes both AKH I and AKH II. This homogeneity makes the CC an excellent system in which to study aspects of neuropeptide biosynthesis. This report summarizes recent findings on AKH inactivation and metabolism, as well as on AKH prohormone processing and biosynthesis.
Key words. Corpus cardiacum; neuropeptide; adipokinetic hormone; prohormone processing; *Schistocerca gregaria*.