Peptides 94 (2017) 1–9

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

Identification of mature peptides from *pban* and *capa* genes of the moths *Heliothis peltigera* and *Spodoptera littoralis*

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ARTICLE INFO

Keywords:
Insect neuropeptide
Transcriptome
Immunocytochemistry
Mass spectrometry
Mimetics
Pyrokinin

ABSTRACT

By transcriptome analysis, we identified PBAN and CAPA precursors in the moths *Spodoptera littoralis* and *Heliothis peltigera* which are among the most damaging pests of agriculture in tropical and subtropical Africa as well as in Mediterranean countries. A combination of mass spectrometry and immunocytochemistry was used to identify mature peptides processed from these precursors and to reveal their spatial distribution in the CNS. We found that the sites of expression of *pban* genes, the structure of PBAN precursors and the processed neuropeptides are very similar in noctuid moths. The sequence of the diapause hormone (DH; tryptopyrokinin following the signal peptide), however, contains two N-terminal amino acids more than expected from comparison with already published sequences of related species. *Capa* genes of *S. littoralis* and *H. peltigera* encode, in addition to periviscerokinins, a tryptopyrokinin showing sequence similarity with DH, which is the tryptopyrokinin of the *pban* gene. CAPA peptides, which were not known from any noctuid moth so far, are produced in cells of abdominal ganglia. The shape of the release sites of these hormones in *H. peltigera* represents an exceptionally derived trait state and does not resemble the well-structured abdominal perisympathetic organs which are known from many other insects. Instead, axons of CAPA cells extensively ramify within the ventral diaphragm. The novel information regarding the sequences of all mature peptides derived from *pban* and *capa* genes of *H. peltigera* and *S. littoralis* now enables a detailed analysis of the bioactivity and species-specificity of the native peptides, especially those from the hitherto unknown *capa* genes, and to explore their interactions with PBAN/DH receptors.

1. Introduction

Neuropeptides are endogenous messenger molecules with a crucial role in controlling and modulating physiological functions of multicellular organisms. Following expression of a neuropeptide gene, translated precursor molecules are subsequently processed and post-translationally modified in the endoplasmic reticulum and the Golgi apparatus. Thereby a single precursor often yields several mature neuropeptides (multiple copy peptides = paracopies) which potentially activate identical receptors or might also target different receptors. In insects, the latter scenario is rare but confirmed *e.g.* for products of the *capa*-gene [1,2]. The *capa* genes of insects share a common evolutionary history with the *pk/pban* (*pyrokinin/pheromone biosynthesis activating neuropeptide*) gene; the predicted duplication of an ancestor gene possibly occurred in a subgroup of arthropods prior to the emergence of insects from this lineage [3]. It can be expected, that in most insects different peptides of *pk/pban* potentially also activate two receptor types; PK/PBAN receptors (PK 2 receptors in *Drosophila* [4]) and a receptor which recognizes tryptoPKs (PK 1 receptor in *Drosophila*; [2]). TryptoPKs (WFGPRLamides; for designation see [5]) are originally encoded on *capa* as well as *pk/pban* genes.

In moths, which contain a number of very harmful pest insects in agriculture, one of the most studied neuropeptide families is the PK/PBAN family, hereafter named just PBAN when referring to moth species [6–9]. The first sequenced representative of this family, leucopyrokinin, was described as myostimulatory peptide from the cockroach *Rhyparobia* (syn. *Leucophaea*) *maderae* [10]. Few years later, a further peptide with C-terminal FXPRLamide was described as PBAN from the corn earworm *Helicoverpa zea*, referring to the stimulation of pheromone biosynthesis in adult moth females [11]. Subsequent accumulation of genome and transcriptome data from insects has disclosed that genes named *hugin* (*Drosophila melanogaster*, [12]), *pban* (Lepidoptera),

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http://dx.doi.org/10.1016/j.peptides.2017.05.004
Received 3 March 2017; Received in revised form 5 May 2017; Accepted 6 May 2017
Available online 11 May 2017

and *pyrokinin* (remaining insects) are homologous and the respective mature products therefore evolved from common ancestor sequences. The PBAN precursor of moths contains, in addition to PBAN, three FXPR/KLamide paracopies which are, for historical reasons, named α-, β- and γ-SGNPs (subesophageal ganglion neuropeptides). The designation SGNP refers to the major expression site of *pban* which are neuroendocrine cells of the subesophageal ganglion (SEG; [13–15]). A major projection of these ventral neurons is into the maxillary nerve and retrocerebral complex which serve as storage and release sites of products derived from the *pban* gene. The *pban* gene also encodes a single tryptoPK, which induces egg diapause in *Bombyx mori* [16]; and terminates pupal diapause in *Heliothis armigera* [17]; it is also known as diapause hormone (DH). The DH is the first peptide in the precursor sequence after the signal peptide [18]. Since the *capa* gene of insects encodes a sequence-related tryptoPK as plesiomorphic character, it can be hypothesized that the CAPA-tryptoPK and DH of moths activate the same receptor and, hence, share the same biological functions [3]. However, functional assays, testing native CAPA-tryptoPKs in moth species or other lepidopterans, have not been reported so far. As described for *pk/pban* above, insect's *capa* gene is also expressed in neuroendocrine cells but this *capa* expression is mainly restricted to neurons in abdominal ganglia. Processed CAPA-peptides are accumulated and likely also released from abdominal perisympathetic organs (aPSOs [19]). In addition to the tryptoPK, a second type of receptor ligand is encoded by *capa*, the periviscerokinins (PVKs). These peptides are known to regulate Malpighian tubule and visceral muscle activity in a number of insects [19,20]. The functions of these peptides were also not yet tested in moths.

The aim of the present study was to identify species-specific products of *pban* and *capa* in the moth species *Spodoptera littoralis* and *Heliothis peltigera*. This will enable to study their bioactivity in the respective moth species and compare, for the first time, the activity of the native peptides with those of other species, in order to provide a better insight into the ligand-receptor interactions and help understand the mode of action of the multifunctional and ubiquitous peptides. *S. littoralis* and *H. peltigera* are among the most damaging lepidopteran pests of agriculture in tropical and subtropical areas of Africa and Mediterranean countries; the larvae feed on a broad variety of plants. Due to global warming, *S. littoralis* and *H. peltigera* are currently spreading northwards and become a serious threat also in other European countries and thus, there is an urgent need for their management. The vital functions mediated by PBAN and CAPA-peptides in these moths render them an important target for the development of novel insect-control strategies based on interference with their activity. Indeed, in the past years several cyclic and linear peptides have been designed and tested for antagonistic PBAN activity in *S. littoralis* and *H. peltigera* moth, which resulted in inhibition of key physiological functions [21–23]. Information about native hormone sequences therefore might support developing taxon-specific peptidomimetics, that target fundamental physiological functions, and thereby controlling the proliferation of these pests without affecting beneficial insects [7,8,24].

2. Material and methods

2.1. Insect species and rearing

S. littoralis and *H. peltigera* larvae were reared on an artificial diet as described in [9]. Once larvae reached the pupal stage, they were sexed and placed in separate rooms with day/light regime of 10/14 h at 25 ± 2 °C and 60–70% relative humidity. Adults were kept in screen cages and fed with 10% sugar solution. Moth populations were refreshed every year with males caught in the field using pheromone traps as described in [9]. All males and females used for our analyses were between two and seven days old.

2.2. Transcriptome analysis

2.2.1. RNA extraction

Total RNA was extracted from adults (*H. peltigera*) and larvae (*S. littoralis*). Animals were kept at 4 °C for half an hour before dissecting the central nervous system (CNS) including brain, SEG, thoracic and abdominal ganglia. CNS samples were preserved in RNAlater solution (QIAGEN GmbH, Hilden, Germany) at 4 °C, with a ratio RNAlater:tissue of approximately 100:1. Total RNA was isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA) following the manufactory instructions at Beijing Genomics Institute (BGI, China). RNA quality parameters were estimated using RNA concentration (ng/μL), RNA integrity number (RIN) and 28S/18S as implement in Agilent 2100 Bioanalyzer.

2.2.2. Library construction and transcriptome sequencing

Libraries were sequenced using the Illumina Truseq RNA Sample Preparation Kit (Illumina, San Diego, USA) at BGI. Briefly, once total RNA was extracted, magnetic beads with Oligo (dT) were used to isolate mRNA and mixed with buffer for fragmenting mRNA into short fragments. The cDNA was synthesized using these mRNA fragments as templates. First-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis and subsequently double stranded cDNA was purified and resolved using elution buffer for end reparation, and Poly (A) tail addition. Finally, after ligating the Illumina sequencing paired ends (PE) adaptors, products were purified on Tris–acetate–ethylenediamine tetraacetic acid agarose gel and suitable fragments were selected for PCR amplification in order to enrich the purified cDNA template. Quality control of the cDNA library was performed using Agilent 2100 Bioanalyzer. Finally, the resulting cDNA library was sequenced on Illumina HiSeqTM4000 with strategies of 100 bp PE at BGI.

2.2.3. De novo assembly

Raw data were initially filtered by removing adapters, reads with more than 5% of unknown bases and reads with low quality sequences (reads having more than 20% bases with quality value lower or equal to 10). Subsequently transcripts were *de novo* assembled using Trinity [25,26]; and Bridger [27] with default options.

2.2.4. Blast search

Pban and *capa* transcripts were searched using amino acid sequences as query for tBLASTn on a local computer as implement in BLAST+ [28]. Identified sequences were translated into proteins using ExPASy translate tool (Artimo et al., 2012; http://web.expasy.org/translate/). Signal peptides were predicted using the SignalP 4.1 server ([29], www.cbs.dtu.dk/services/SignalP/). Cleavage sites were manually assigned based on known PBAN and CAPA peptides [6,8,9,30].

2.3. Mass spectrometry

2.3.1. Sample preparation for mass spectrometry

For direct tissue profiling by MALDI TOF mass spectrometry, parts of the central nervous system, nerves and neurohemal organs from larvae and adults were dissected in insect saline solution with the following composition: 7.50 g/l NaCl, 0.20 g/l KCl, 0.20 g/l $CaCl_2$, 0.10 g/l, $NaHCO_3$ (pH 7.4). Subsequently, isolated samples were transferred into a drop of purified water on a stainless steel sample plate for MALDI-TOF mass spectrometry.

For Orbitrap mass spectrometry analyses, the CNS was dissected, transferred in a drop of insect saline for removing attached fat body and trachea. The samples were then transferred in 30 μl extraction solution (50% methanol, 49% H_2O,1% formic acid [FA]). Tissues and cells were disintegrated using an ultrasonic bath (Transonic 660/H, Elma Schmidbauer GmbH, Hechingen, Germany) for 5 min on ice and an ultrasonic-probe three times for 5 s (BandelinSono HD 200, Bandelin