Insect Biochemistry and Molecular Biology 39 (2009) 430–439

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

A lepidopteran pacifastin member: Cloning, gene structure, recombinant production, transcript profiling and *in vitro* activity

Bert Breugelmans [a,*], Gert Simonet [a], Vincent van Hoef [a], Sofie Van Soest [a], Guy Smagghe [b], Jozef Vanden Broeck [a]

[a] Department of Animal Physiology and Neurobiology, Zoological Institute K.U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium
[b] Laboratory of Agrozoology, Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

ARTICLE INFO

Article history:
Received 24 December 2008
Received in revised form
20 March 2009
Accepted 23 March 2009

Keywords:
Inhibitor
Peptide
Pacifastin
Serine peptidase
Bombyx mori
Gene structure

ABSTRACT

Members of the pacifastin family have been characterized as serine peptidase inhibitors (PI), but their target enzyme(s) are unknown in insects. So far, the structural and biochemical characteristics of pacifastin-like PI have only been studied in locusts. Here we report the molecular identification and functional characterization of a pacifastin-like precursor in a lepidopteran insect, i.e. the silkworm *Bombyx mori*. The *bmpp-1* gene contains 17 exons and codes for two pacifastin-related precursors of different length. The longest splice variant encodes 13 inhibitor domains, more than any other pacifastin-like precursor in arthropods. The second transcript lacks two exons and codes for 11 inhibitor domains. By studying the expression profile of the *Bombyx* pacifastin-like gene a different expression pattern for the two variants was observed suggesting functional diversification. Next, several PI domains of BMPP-1 were produced and, contrary to locust pacifastin peptides, they were found to be potent inhibitors of both bovine trypsin and chymotrypsin. Surprisingly, the same *Bombyx* PI are only weak inhibitors of endogenous digestive peptidases, indicating that other peptidases are the *in vivo* targets. Interestingly, the *Bombyx* PI inhibit a fungal trypsin-like cuticle degrading enzyme, suggesting a protective function for BMPP-1 against entomopathogenic fungi.

1. Introduction

According to the MEROPS database (http://merops.sanger.ac.uk) (Rawlings et al., 2008) members of the pacifastin family (I19) are inhibitors of the largest family of serine peptidases (S1 family). All pacifastin members that have been characterized at the molecular level are precursor peptides composed of an N-terminal signal sequence followed by a variable number of inhibitor domains. These domains are designated as PLDs (Pacifastin Light chain Domains) in reference to the light chain of pacifastin, the first member of the pacifastin family that was found in the crustacean species, *Pacifastacus leniusculus* (Hergenhahn et al., 1987; Liang et al., 1997). All other pacifastin-related precursors (PPs) that have been identified by cDNA cloning originate from only two insect orders: Orthoptera and Hymenoptera (Kromer et al., 1994; Parkinson et al., 2002; Simonet et al., 2002a, 2002b, 2004a, 2004b, 2005) and only in locusts, the structural and biochemical characteristics of pacifastin-related inhibitors have been intensively studied by ^{1}H NMR, crystallography, site directed mutagenesis and activity studies (Boigegrain et al., 1992; Mer et al., 1994, 1996; Kellenberger et al., 1995; Hamdaoui et al., 1998; Malik et al., 1999; Roussel et al., 2001; Gaspari et al., 2002; Simonet et al., 2005; Fodor et al., 2005). However, previous *in silico* data mining studies have predicted additional pacifastin members in various other insect orders (Simonet et al., 2003a; Gaspari et al., 2004; Breugelmans et al., 2008b).

Conforming to the presence of putative dibasic cleavage sites between inhibitor domains, nearly all identified insect PPs are believed to be processed into smaller inhibitor peptides (Kellenberger et al., 1995; Hamdaoui et al., 1998; Simonet et al., 2002a, 2002b, 2004b, 2005). All PLD-related domains are characterized by a conserved pattern of six cysteine residues (**Cys1** – Xaa_{9-12} – **Cys2** – Asn – Xaa – **Cys3** – Xaa – **Cys4** – Xaa_{2-3} – Gly – Xaa_{3-6} – **Cys5** – Thr – Xaa_{3} – **Cys6**). Detailed analysis of the 3-D structure shows that these six residues form three disulfide bridges (Cys1–4, Cys2–6, Cys3–5), giving members of the pacifastin family a typical fold and remarkable stability (Mer et al., 1994, 1996; Roussel et al., 2001; Gaspari et al., 2002; Kellenberger et al., 2003).

Each pacifastin-like inhibitor domain contains a reactive site (P1–P1′) that is involved in the binding of the target peptidase and inhibitor activity studies on locust pacifastin-like peptides have

* Corresponding author. Tel.: +3216324260; fax: +3216323902.
E-mail address: bert.breugelmans@bio.kuleuven.be (B. Breugelmans).

0965-1748/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ibmb.2009.03.005

confirmed that the peptidase specificity is mainly determined by the residue at the P1-position (Kellenberger et al., 1995; Hamdaoui et al., 1998; Malik et al., 1999; Roussel et al., 2001; Gaspari et al., 2002; Simonet et al., 2005). Although all available data indicate that members of the pacifastin family share a common conformation, the family can be divided into at least two separate groups (I & II) because of different intramolecular interactions in the inner core region (Kellenberger and Roussel, 2005). The variability in the reactive site (P1–P1′), the inner core structure and additional peptidase interaction sites contribute to a different peptidase specificity and species selectivity between members of the two groups.

Contrary to the detailed knowledge on both the structure and the inhibitory activity of locust pacifastin-like members, very little is known about their *in vivo* function(s). In crayfish, pacifastin itself is involved in the regulation of an immune response related serine peptidase-dependent cascade (the prophenoloxidase cascade) (Liu et al., 2007). In insects, on the other hand, no direct evidence is available on the possible function(s) of the pacifastin-related. Detailed transcript profiling studies (Northern blot experiments and real-time PCR studies) on locust pacifastin-related precursors led to a number of hypotheses about possible functions in insects (Vanden Broeck et al., 1998; Simonet et al., 2002a, 2004a, 2004b, 2005; Franssens et al., 2008; Breugelmans et al., 2008a). These profiling studies showed that the locust PP transcripts are differentially regulated in (i) a variety of tissues (fat body, brain, gonads, ventral nerve cord, foregut and hindgut), (ii) during development (larval stages, in adults), (iii) between genders, (iv) depending on the phase (gregarious *versus* solitarious) and (v) upon immune challenge with fungal components. Therefore, pacifastin-related inhibitors are believed to have multiple functions in locusts as regulators of diverse serine peptidase-dependent processes (Hamdaoui et al., 1998; Malik et al., 1999; Simonet et al., 2003b). In this study, a pacifastin-like member of the order of the Lepidoptera was cloned from the silkworm, *Bombyx mori*. In addition, the structure as well as the expression profile of the *Bombyx* pacifastin-like gene was studied. Furthermore, *Bombyx* pacifastin-related precursor domains were produced and the *in vitro* inhibitory activity of these multi-headed PI was analyzed.

2. Methods

2.1. Experimental organisms

Larvae of the silkworm, *B. mori* (Daizo strain), were reared on fresh mulberry leaves at 25 °C under 12 L:12 D photoperiod. In addition, an entomopathogenic fungus, *Beauveria bassiana* was grown and stimulated to secrete cuticle degrading enzymes (Donatti et al., 2008).

2.2. Cloning of the Bombyx pacifastin-related precursor

2.2.1. mRNA extraction and cDNA synthesis

Decapitated bodies of last larval silkworms were added to reaction tubes containing 'Green Beads' (Roche, Indianapolis, IN, USA). Then, semi-automated homogenization of the samples was performed in the 'MagNA Lyser' (Roche) instrument according to the manufacturer's instructions. Subsequently, total RNA was extracted from the homogenates utilizing the 'RNeasy Lipid tissue mini kit' (Qiagen, Valencia, CA, USA). The resulting RNA was reverse transcribed (M-MLV RT, Invitrogen Life Technologies) utilizing random hexamers as described in the provided protocol.

2.2.2. Primer design and polymerase chain reaction (PCR)

Different sets of gene specific primers were designed based on PP-encoding EST sequences. The sequences of the primers (SIGMA) are presented in Table 1. Restriction sites were added to particular primers allowing for a directional ligation of the amplification of fragments into an expression vector (*cf.* 2.4).

Table 1
Overview of gene specific primers. Different sets of primers are designed to clone the ORF of the pacifastin-related precursor in *B. mori*. The sequences of the restriction sites are underlined and stop codons are in bold. Primers corresponding to a specific variant of BMPP-1 are named accordingly.

Construct	Primer	Primer sequence	Restriction site
	U1	5′-AAACAGTGTTGTGATTGAGGATTG-3′	
	D1	5′-TGGC**TTA**TGCGGCGTTT-3′	
	U2	5′-GATAACGGTCTGGGCCTATGCTC-3′	
	D2a	5′-CAGGGTGTGTTAGGTATTTCGGT-3′	
	D2b	5′-CGCGACGCAAACGATTGCTT-3′	
	U3a	5′-ACCGAAATACCTAACCACACCCTG-3′	
BMPP-C	U3b	5′-GATATCGTTTGCGTCGCGAACC-3′	EcoRV
	D3	5′-AAGCTTGGC**TTA**TGCGGCGTTTATG-3′	HindIII
BMPP-N	U	5′-GAAGAAATTCGGAAGTGGAATGCCTGGC-3′	XmnI (PdmI)
	D	5′-AAGC**TTA**GATCAGCTCAGACTTCTCGGGAG-3′	HindIII

PCRs (50 μl) contained 5 μl 10× PCR Buffer (provided by the manufacturer), 1 μl of a 10 mM dNTP mix, 1 μl of each primer (10 μM), 2 μl whole body cDNA and 0.2 μl platinum Taq DNA polymerase (Invitrogen Life Technologies). Hot-start PCR was run for 35 cycles in a 'triothermoblock TB-1' thermocycler (Biometra). Each cycle consisted of a denaturation step for 1 min at 94 °C, an annealing step for 1 min at a specific temperature according to the selected primer set and an extension step for 1 min at 68 °C, with a final extension step of 7 min at 68 °C. PCRs were analyzed by horizontal agarose gel electrophoresis. The specific PCR fragments were subcloned and sequenced as outlined below.

2.2.3. Cloning, sequencing and sequence analysis

The PCR products were separated on an agarose gel by electrophoresis and purified with the GenElute Gel Extraction Kit (Sigma). The purified PCR fragments were then subcloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen Life Technologies). After plasmid isolation (GenElute HP Plasmid Miniprep Kit: SIGMA), the inserts were sequenced on the 3130 Genetic Analyzer (Applied Biosystems) and all nucleotide and deduced amino acid sequences were compared with the AlignX software (InForMax, Inc., Invitrogen Life Technologies). To study the intron–exon organization of the *Bombyx* pacifastin gene in more detail, EST and cDNA sequences were aligned with the genome sequence using Splign (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).

2.3. Real-time RT-PCR analysis

2.3.1. Experimental samples

In order to study the transcript distribution of pacifastin-related precursor transcripts (BMPP-1a and BMPP-1b) in Lepidoptera, *B. mori* larvae were synchronized and at days 2 and 6 of the last larval stage different tissues were dissected (10 animals per pooled sample; three independent samples per condition): foregut (Fg), midgut (Mg), hindgut (Hg), Gonads (Gon), head (H), ventral nerve cord (VNC), silk glands (SG) and tracheae (Trach). In addition, fat body (Fb) was dissected at day 6 of the last larval stage. All insect tissues were microdissected under a binocular microscope and immediately collected in RNA*later* (Ambion) solution to prevent degradation. Until further processing, these pooled tissue samples were stored at −20 °C.

2.3.2. Total RNA extraction and cDNA synthesis

The total RNA samples were prepared as described above (2.2.1). In combination with this extraction procedure, a DNase treatment