Phylogenetic distribution of protease inhibitors of the Kazal-family within the Arthropoda

Vincent van Hoef*, Bert Breugelmans, Jornt Spit, Gert Simonet, Sven Zels, Jozef Vanden Broeck

Department of Animal Physiology and Neurobiology, Zoological Institute K.U.Leuven, Naamsestraat 59, PO Box 02465, B-3000 Leuven, Belgium

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ABSTRACT

In mammalian pancreatic cells, the *pancreatic secretory trypsin inhibitor* (PSTI) belonging to the Kazal-family prevents the premature activation of digestive enzymes and thus plays an important role in a protective mechanism against tissue destruction by autophagy. Although a similar protective mechanism exists in Arthropoda, the distribution of these inhibitors in this phylum remains obscure. A comprehensive in silico search of nucleotide databases, revealed the presence of members of the Kazal-family in the four major subphyla of the Arthropoda. Especially in the Hexapoda and the Crustacea these inhibitors are widespread, while in the Chelicerata and Myriapoda only a few Kazal-like protease inhibitors were found. A sequence alignment of inhibitors retrieved in the digestive system of insects revealed a conservation of the PSTI characteristics and strong resemblance to vertebrate PSTI. A phylogenetic analysis of these inhibitors showed that they generally cluster according to their order. The results of this data mining study provide new evidence for the existence of an ancient protective mechanism in metazoan digestive systems. Kazal-like inhibitors, which play an important protective role in the pancreas of vertebrates, also seem to be present in Arthropoda.

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1. Introduction

A healthy mammalian pancreas delivers its stored inactive digestive enzyme precursors to the gut. Once secreted in the gut lumen, the digestive enzymes are activated in a cascade-like manner, starting with the cleavage of trypsinogen into the active trypsin by enterokinase. Subsequently, trypsin activates the other pro-teases needed for the digestion of proteins in the food [10]. To prevent premature activation of digestive enzymes and the resulting damage to cells and tissues, mammals express a protease inhibitor in their pancreatic cells called ‘pancreatic secretory trypsin inhibitor’ (PSTI) [11,16]. This inhibitor is a member of the numerous and diverse Kazal-family of protease inhibitors and binds to prematurely activated trypsin, interrupting the activation cascade. Typically, a mammalian Kazal-type serine protease inhibitor domain is 40–60 amino acids long, has a molecular mass of approximately 6500 Da and has a conserved domain architecture [3,13]. This Kazal domain or motif has a general amino

acid sequence of Cys_I-X_a-Cys_{II}-X_b-PVCys_{III}G-X_c-TY-X_d-Cys_{IV}-X_e-Cys_V-X_f-G-X_g-Cys_{VI} where the subscripts a, b, c, d, e, f and g are integral numbers of amino acid residues. The six cysteine residues form three intra-domain disulfide bridges in a characteristic pattern (Cys_I-Cys_V, Cys_{II}-Cys_{IV} and Cys_{III}-Cys_{VI}) contributing to a tight three-dimensional conformation. The second amino acid C-terminal to the second cysteine is the important specificity determining P1-residue (see Fig. 2) [31].

The ‘authentic’ PSTI occurs only in vertebrate species [1,8,11,23,24,41], which is logical since they are the only animal group with an actual pancreas. However, a study in 2006 revealed the presence of the PSTI-homolog Kazal1 in the endodermal cell layer of the body column of the freshwater polyp, *Hydra magnipapillata* [6]. The cells expressing Kazal1 showed the specific morphology of gland cells, with a multi-vacuolated cytoplasm corresponding to large secretory vesicles. Considering the similar structure and cellular localization, the authors speculated that Kazal1’s function was to prevent the premature activation of digestive enzymes. An RNAi-mediated knockdown of this inhibitor resulted in severe autophagy of gland cells and epithelial digestive cells. This phenotype resembles pancreatitis – a disease which is associated with dysfunctional PSTI in vertebrates. So, even though *Hydra* does not possess a pancreas, a similar protease inhibitor seems to have the same protective role as PSTI has in vertebrates. Cnidaria – the phylum *Hydra* belongs to – are an evolutionary sister group to the Bilateria (including vertebrates and arthropods),

* Corresponding author. Tel.: +32 16324260; fax: +32 16323902.

E-mail addresses: Vincent.vanHoef@bio.kuleuven.be, vvanhoef@hotmail.com (V. van Hoef), Bert.Breugelmans@bio.kuleuven.be (B. Breugelmans), Jornt.Spit@bio.kuleuven.be (J. Spit), Gert.Simonet@bio.kuleuven.be (G. Simonet), Sven.Zels@bio.kuleuven.be (S. Zels), Jozef.VandenBroeck@bio.kuleuven.be (J. Vanden Broeck).

suggesting an ancient origin for this protective mechanism in a common ancestor. Since Arthropoda emerged only after the divergence of Cnidaria and Bilateria more than 580 Mya [5], a comparable defensive role for PSTI-like proteins in arthropods seems plausible. Further evidence for a similar function in arthropods was observed in the locust *Locusta migratoria* [36]. Again, an RNAi-mediated knockdown of a locust-specific PSTI-like protein (LmPSTI) resulted in a phenotype not unlike that observed in mammals suffering from pancreatitis (i.e. the occurrence of autophagy in the insect's caeca).

This manuscript reports on a detailed, comparative in silico data mining study of PSTI-like protein precursors among Arthropoda – with a special emphasis on the distribution in Insecta. To our knowledge, no arthropod PSTI-homologs have been characterized as such so far (except for LmPSTI in *L. migratoria*). However, there have been numerous reports of Kazal-type family protease inhibitors (PSTI being an important member of the Kazal-type family inhibitors) with a variety of suggested functions in various arthropod classes and orders including species, such as *Bombyx mori* [42], *Rhodnius prolixus* [9], *Aedes aegyptii* [39] and several crustaceans [4,7,19,38].

2. Materials and methods

2.1. Database searches

Arthropod homologs of PSTI were searched for in silico in EST (Expressed Sequence Tags) databases using a list of 15 known vertebrate PSTI precursor sequences from the Uniprot protein database (<http://www.uniprot.org>) as a query (see additional data). To check and further complement previous search results, novel arthropod sequences were used as additional queries. Detailed searches were performed on a vast number of nucleotide sequences available via the BLAST (Basic Local Alignment Search Tool) function accessible on the NCBI platform (<http://www.ncbi.nlm.nih.gov/BLAST/>). Complementary to this, various databases of individual species containing new sequences that are not yet incorporated into the NCBI platform were analyzed (BeetleBase; beetlebase.org, FlyBase; flybase.org).

2.2. Strategy

The data mining search was performed in a systematic way, covering as many classes and orders as possible. Per arthropod subphylum, EST sequence data sets were analyzed for the presence of PSTI homologs and, whenever this was possible, additional searches were performed in the classes, orders and families of the subphylum. Since Kazal-like inhibitors (the peptide family PSTI belongs to) are a very numerous family with many and diverse functions, a distinction was made between EST sequences originating from the organisms' digestive system and others. This distinction was made to focus on the novel inhibitors that potentially have a similar role as the mammalian PSTI – namely to protect the organism from damage due to prematurely activated digestive enzymes.

2.3. Sequence comparison and phylogenetic analysis of insect PSTI-like proteins

The PSTI-like precursors obtained from insect EST sequences originating from the digestive system were restricted in length to the two exterior Cys residues (Cys_I to Cys_{VI}) and aligned using ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and visualized in the perl/tk-based tool Aline (<http://crystal.bcs.uwa.edu.au/px/charlie/software/aline/>). The alignment file which resulted from the ClustalW alignment was used as an input file to visualize the phylogeny of the PSTI-like precursors in the free software program PHYLIP (PHYLogeny Inference Package)

(<http://evolution.genetics.washington.edu/phylip.html>) using the maximum parsimony method.

3. Results

By almost any measure, arthropods are the most diverse and successful group of animals on the planet. They make up over three quarters of all known currently living and fossil organisms, and are represented by – according to scientists' recent estimates – 3.7 million species [14]. So far, however, 'only' around 855,000 species have been described, which leaves approximately 75% of the contemporary arthropod species awaiting scientific description. This large number of species is divided into four subphyla: Myriapoda (centipedes, millipedes, etc.), Chelicerata (horseshoe crabs, spiders and pycnogonids), Crustacea (shrimps, crabs, etc.) and Hexapoda (insects and springtails). As described in Section 2, a similarity search was performed to discover novel Kazal-like inhibitors and potential PSTI homologs. The results are visualized in Fig. 1.

3.1. PSTI-like inhibitors in Myriapoda

Myriapoda is a subphylum of the Arthropoda containing among others the centipedes and the millipedes (hence the name Myriapoda, which means 10,000 legs in Greek). They are subdivided in four subphyla: the Chilopoda, the Diplopoda, the Pauropoda and the Symphyla. Genetic data of this animal group are scarce, with only 4461 EST sequences and 3893 nucleotide sequences submitted to the NCBI platform (and almost completely confined to the diplopodans). A similarity search through EST data, revealed one significant hit in the African giant black millipede, *Archispirostreptus gigas*. Since the *A. gigas* EST sequences originated from a whole animal, a further tissue distribution was not available. Although several Kazal-like inhibitor features are present (6 cysteines, conserved amino acids) and the P1-residue is a Lys (suggesting trypsin inhibitory specificity), other characteristics differ from a typical Kazal-domain (most notably, a remarkably short amino acid stretch between Cys_V and Cys_{VI}). In the Chilopoda, Pauropoda and Symphyla, no PSTI-homolog could be discovered.

3.2. PSTI-like inhibitors in Chelicerata

The subphylum of Chelicerata is currently subdivided in three classes: the Arachnida (arachnids), the Pycnogonida (sea spiders) and the Merostomata (horseshoe crabs). Genetic data about this subphylum are more abundant in comparison with data about Myriapoda – especially the Arachnida are well-represented. Searching this relative abundance of nucleotide information did, however, not result in a large number of Kazal-like inhibitors. In the Southeast Asian horseshoe crab, *Carcinoscorpius rotundicauda* (a member of the Merostomata), and the Black-legged tick, *Ixodes scapularis* (a member of the Arachnida), respectively two and one putative Kazal-like inhibitors were found. Not surprisingly, these two species are greatly overrepresented in the nucleotide information available about their class (46.5% and 93.5% for *Ixodes* and *Carcinoscorpius*, respectively). A further localization in the digestive system could not be deduced for these inhibitors. A search through the very recently sequenced genome of the spider mite, *Tetranychus urticae* revealed the presence of two putative Kazal-type inhibitors [12]. Searching through currently available pycnogonid nucleotide information did not yield potential Kazal-type inhibitors.

3.3. PSTI-like inhibitors in Crustacea

Crustacea is a large and diverse subphylum of the Arthropoda, including many commercially (and gastronomically) important

Fig. 1. Distribution of Kazal-like protease inhibitors in Arthropoda. Orders and classes for which less than 1000 EST sequences are deposited on the NCBI platform are marked by an *. The classes and orders in which Kazal-type inhibitors are identified are highlighted with a box and the classes and orders where one or more inhibitors are located in the digestive system are marked in gray.

species, such as the common freshwater shrimp (*Gammarus pulex*), the Pacific white shrimp (*Litopenaeus vannamei*) and the American lobster (*Homarus americanus*). A lot of sequence data are available for these species and several genome projects are generating a growing amount of information. Three classes of the subphylum Crustacea were examined for the presence of Kazal-like protease inhibitors and possible PSTI homologs: the class of Branchiopoda (comprises fairy shrimp, clam shrimp and Cladocera), the class of Maxillopoda (includes barnacles and copepods) and the class of

Malacostraca (displays a greater diversity in body forms than any other animal class and includes species such as crab, lobster, krill and shrimp).

In the NCBI platform, Branchiopoda are mainly represented by *Daphnia* species of the order of Diplostraca (around 172,000 ESTs). An earlier search in the draft assembly of the genome of *Daphnia pulex* had already shown the presence of at least 35 Kazal-type inhibitor domains [4]. Our similarity search in the EST sequences indeed revealed several putative Kazal-like inhibitors.

Their (possible) localization in the digestive tract could however not be deduced from the nucleotide data. The order of Notostraca (shield shrimps and tadpole shrimps) also appears to contain at least one Kazal-like inhibitor in *Triops cancriformis*, despite the relative scarcity of EST information. Anostraca (fairy shrimps) do not appear to contain Kazal-like inhibitors, despite being relatively well represented in the EST databases.

The class of Maxillopoda contains many orders (in Fig. 1 only the orders for which EST data was available are represented), but only in the Harpacticoida and Siphonostomatoida putative Kazal-like inhibitors were present.

The large and diverse class of Malacostraca possesses Kazal-like inhibitors in three orders: Isopoda (pill bugs, wood lice and sea slaters), Euphausiacea (krill) and Decapoda. In the Isopoda one homolog could be found in *Euridice pulchra*. Interestingly, this inhibitor seems to lack Cys_{II} and Cys_{IV} (resulting in two disulfide bridges instead of the normal three). This kind of 'atypical Kazal domains' has been described before in several invertebrate species, such as *Galleria mellonella* [27], *Argopecten irradians* [43] and *Chlamys farreri* [37]. In the EST information of the Euphausiacea, a double domain Kazal-like inhibitor was retrieved in the head (but not the digestive tract) of *Euphasia superba*. Earlier studies had already revealed the presence of many Kazal-like inhibitors in the order of the Decapoda. For example in *Penaeus monodon* [33], *L. vannamei* [18], *Pacifastacus leniusculus* [19] and *Procambarus clarkia* [40] multiple domain Kazal-like inhibitors have been identified. They are important in several biological processes, such as reproduction, protection against pathogen-derived proteinases and antimicrobial activity. A multi-domain Kazal-like inhibitor from *L. vannamei* was also found in the organism's hepatopancreas. This organ appears in the digestive tract of certain arthropods, mollusks and fish and is responsible for the functions which are provided separately by the liver and the pancreas in mammals. Several studies have shown that this organ stores and secretes digestive enzymes, such as serine proteases [20,21,34]. In this case, Kazal-type protease inhibitors native to the hepatopancreas might play a role in preventing cellular damage after premature enzyme activation.

3.4. PSTI-like inhibitors in Hexapoda

The subphylum of Hexapoda is the largest (in terms of number of species) grouping of arthropods and includes the classes of Ellipura (springtails), Diplura (two-pronged bristletails) and Insecta. Their name was derived from an important common feature: a consolidated thorax with three pairs of legs (in contrast to other arthropods, most of which have more than three pairs of legs). Insects in particular are highly rich in species and represent more than half of all known living organisms. This diversity and their economic importance – both beneficial and destructive – are reflected in the amount of nucleotide data available: 76% of the arthropod EST sequences on the NCBI platform originate from the class of Insecta.

In Ellipura, Kazal-type inhibitors were found in two species of the order of Collembola: *Folsomia candida* and *Onychiurus arcticus* (Arctic springtail). No information on their tissue distribution is available.

In Diplura, no Kazal-type inhibitors were retrieved, possibly due to the current shortage of available sequence data for this class.

In insects, several previous studies have shown the presence of Kazal-like inhibitors in species, such as *B. mori* [42], *Drosophila melanogaster* [26], *A. aegyptii* [30], *Triatoma infestans* [22] and *R. prolixus* [9]. The biological and physiological functions they perform are diverse. For example, blood-sucking insects, such as *R. prolixus* and *T. infestans*, produce Kazal-type proteinase inhibitors (rhodniin and infestin, respectively) which display a strong affinity for thrombin and play an important role as anticoagulant during

the ingestion of a blood meal. On the other hand, in moths, such as *B. mori*, a Kazal-type inhibitor is produced in the silk glands and is present in the cocoon silk besides the major silk proteins. The presence of this serine protease inhibitor and its activity against the bacterial subtilisin and fungal proteinase K suggests a function in the protection of silk cocoon against degradation by microbial proteinases [42]. For other inhibitors the function remains to be elucidated.

Our search in insect EST-databases revealed additional potential Kazal-type inhibitors in the majority of insect orders. These inhibitors seem to be present throughout the class of Insecta: from hemimetabolous orthopteran, blattarian and isopteran species to holometabolous hymenopteran, lepidopteran, dipteran, mecopteran and siphonapteran species. In the order of Orthoptera, Kazal-type inhibitors are found in the two major suborders: Caelifera (locusts and grasshoppers) and Ensifera (crickets and katydids). In Hemiptera, Kazal-like inhibitors are found in all three important clades, i.e. the clade of the true bugs (Heteroptera), the cicadas (Auchenorrhyncha) and the aphids (Sternorrhyncha). The presence of these inhibitors in phloem-feeding aphids raises some interesting questions concerning their function, since these organisms are believed to have only marginal proteolytic digestion by proteases (and mainly by cysteine proteinases). A recent study however, showed that there appears to be significant proteolytic digestion in cereal aphids, allowing for a regulatory role of these inhibitors in the activation of digestive proteases – even in aphids [29]. In six other orders EST-data showed that Kazal-type inhibitors are present in the digestive tract and could therefore have a similar protective role as PSTI-like inhibitors in vertebrates and *Hydra*. These orders were the Orthoptera (grasshoppers and locusts), Hemiptera (bugs), Coleoptera (beetles), Lepidoptera (butterflies and moths), Diptera (flies) and Siphonaptera (fleas). Additional searches in FlyAtlas (fly-atlas.org) revealed that Kazal-like inhibitors are mainly present in the midgut of larval *Drosophila*. This confirms the results of a study by Niimi et al., which locates similar inhibitors in the secretory pathway and the cytosol of the *Drosophila* midgut [26]. A sequence comparison and phylogenetic analysis at the species level of these six orders is presented in the next paragraph.

3.5. Sequence comparison and phylogenetic analysis of insect PSTI-like proteins

By searching the available insect EST-data, 49 new Kazal-like inhibitors were predicted which are expressed in the digestive system and thus are potential PSTI-like peptides. Aligning these with human PSTI revealed several conserved amino acids (see Fig. 2). In all peptides, six cysteine residues are conserved which form a disulfide linkage pattern typical for Kazal-like inhibitors. The specificity determining P1-residue is predominantly Arg or Lys, which is indicative of trypsin inhibitors and in accordance with their suggested function: preventing trypsin from activating the (other) pro-enzymes prematurely. In some cases, however, another residue is found at this site; an often encountered P1-variant is Leu, which suggests an inhibitory preference for chymotrypsin, just like Met, Phe and Tyr. Two species (*R. prolixus* 2 and *O. nubilalis* 3) have an Asp at this position, suggesting an inhibitory activity toward cysteine proteases, which is uncommon for the serine protease targeting Kazal-like inhibitors. Inhibition of cysteine proteases excreted by the bacterium *Porphyromonas gingivalis* by PSTI has been demonstrated [2], but these cysteine proteases – called gingipains – are Lys- and Arg-specific allowing for PSTI to be a suitable substrate and effective inhibitor. For *R. prolixus* 2, however, another role has been suggested. This inhibitor is part of a multi-domain Kazal-type inhibitor (rhodniin) directed toward thrombin; domain 1 interacts with thrombin via the classical method described for Kazal-like inhibitors while domain 2 facilitates the thrombin-rhodniin

Fig. 2. Alignment of human PSTI with Kazal-like inhibitors present in the digestive system of insects. The amino acid sequences are restricted to the two exterior cysteines. The brackets under the consensus sequence represent the typical 1-5/2-4/3-6 disulfide bridge pattern between the Cys-residues. The P1-residue is indicated with an asterisk.

interaction through electrostatic interactions independent of its P1-residue [35]. The role of these non-trypsin inhibitors in stopping the zymogen activation cascade is thus not entirely clear, but one could imagine functions, for instance in preventing damage from already activated chymotrypsin or possibly even cysteine proteases. Although a considerable sequence variation can be observed between the PSTI-homologs, the region between the Pro two positions before Cys_{III} and Cys_V has a higher degree of conservation than the rest of the peptide. Also, a Leu and a Gly residue, situated between Cys_V and Cys_{VI}, are fairly well conserved.

4. Discussion

A similarity search for Kazal-type inhibitors in EST sequences originating from arthropod species revealed that this inhibitor family is present in the four major subphyla. In particular in the Hexapoda and Crustacea, Kazal-like inhibitors seem widespread with members of this family found in all studied crustacean classes and in two out of the three studied hexapodan classes. The absence of these inhibitors in some classes and orders seems merely a consequence of the current scarcity of EST data, rather than a reflection of their true distribution.

The subject of this study is a particular member of the Kazal family, namely PSTI. The detection of PSTI in the EST data is complicated because of their sequence resemblance to other Kazal-type inhibitors and the absence of functionally characterized arthropod PSTI-like inhibitors. Therefore, only inhibitors expressed in the digestive system were upheld for a sequence alignment and phylogenetic analysis of putative PSTI-like peptides from insects. In general, insect Kazal-like protease inhibitors resemble their mammalian counterparts fairly well, except for the number of

amino acids between the first two conserved cysteine residues. In mammals, usually six amino acids occupy the space between Cys_I and Cys_{II}, while in invertebrates this is typically limited to just one or two residues. According to Hemmi et al., this results in a less flexible conformation causing the invertebrate inhibitors to be more (protease) specific [15].

A recent study demonstrated the high sequence variability of Kazal-like inhibitors in Crustacea, obscuring the evolutionary connections [4]. In contrast, Kazal-like inhibitors of the digestive tract in insects appear not to be equally variable. The newly found PI's originated from species of six insect orders – including the largest order: the Coleoptera – and a cladogram shows that they generally cluster together according to their order (see Fig. 3). However, the tree shown in Fig. 3 does not completely reflect relatedness between species or orders. For example, in our analysis lepidopteran PSTI-homologs seem more similar to hemipteran PSTIs, while actually Lepidoptera are nearer to Diptera. Also the fact that LmpSTI is placed among the PSTI-homologs of beetles does not seem to mimic the orthopteran and coleopteran phylogeny.

In many species, multiple isoforms are found. Whether these are the product of gene multiplication, multi-domain inhibitors or the result of alternative splicing remains to be investigated. The functional PSTI-homolog Kazal1 in the freshwater polyp *H. magnipapillata* is a three-domain inhibitor but in vertebrates, PSTI is mostly a single domain inhibitor. In the rat, however, an additional PSTI-like inhibitor is found with a possible function as a monitor peptide which observes food levels in the gut and subsequently regulates CCK secretion [25]. PSTI also has some structural similarities to epidermal growth factor (EGF). They share ±50% amino acid sequence homology and have a similar number of residues and three disulfide bridges [17,32]. In addition, PSTI was shown to

Fig. 3. Cladogram of the retrieved insect PSTI-like sequences. Insect order color code: blue: Lepidoptera; red: Hemiptera; green: Diptera; gray: Coleoptera; black: Orthoptera; yellow: Siphonaptera. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

stimulate the growth of several cell lines and is able to bind the EGF receptor [28]. Therefore, other and divergent functions for some of the isoforms of Kazal-type PSTI-like inhibitors in invertebrates cannot be excluded.

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