Insect Biochemistry and Molecular Biology 41 (2011) 688–695

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Functional analysis of a pancreatic secretory trypsin inhibitor-like protein in insects: Silencing effects resemble the human pancreatic autodigestion phenotype

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ARTICLE INFO

Article history:
Received 25 January 2011
Received in revised form
28 April 2011
Accepted 29 April 2011

Keywords:
Protease inhibitor
Locusta migratoria
Kazal
Autophagy
Digestion
Caeca

ABSTRACT

Introduction: In mammalian pancreatic cells, the *pancreatic secretory trypsin inhibitor* (PSTI) prevents the premature activation of digestive enzymes and thus plays an important role in a protective mechanism against tissue destruction by autophagy, a process which may ultimately cause diseases such as pancreatitis and pancreatic cancer. Insects, however, lack a pancreas and so far no PSTI-like peptides are functionally characterized.

Results: In several insect species protease inhibitors that structurally resemble the mammalian PSTI were predicted *in silico*. A putative PSTI-like protein (LmPSTI) was cloned and sequenced in the African migratory locust, *Locusta migratoria*. For the first time the expression of an insect derived PSTI-like inhibitor was shown to be restricted to the digestive enzyme-producing organs in insects (midgut and caeca). LmPSTI was produced via a bacterial expression system and was found to be a potent inhibitor of bovine trypsin as well as endogenous locust gut enzymes. In the caeca, RNAi-mediated knockdown of *LmPSTI* resulted in a significantly upregulated expression (2-fold) of locust ATG8 transcripts (an ubiquitin-like protein crucial for autophagosome formation). These findings were confirmed by an ultrastructural study on caeca, revealing the presence of autophagy-related structures in RNAi-treated animals.

Conclusion: The results of this study lead us to believe that LmPSTI plays an important role in controlling the proteolytic activity in the digestive system of *L. migratoria*. These findings provide new evidence for the existence of an ancient protective mechanism in metazoan digestive systems and open new perspectives for the study of autophagy-related diseases in the digestive tract.

1. Introduction

Diseases involving the pancreatic system – notably pancreatitis and pancreatic cancer – continue to pose a major public health concern worldwide. In the United States alone, approximately 80 000 people are hospitalized annually due to acute pancreatitis and 40 000 due to chronic pancreatitis, resulting in around 3500 deaths per year (Brown et al., 2008; Everhart and Ruhl, 2009; Gupta and Toskes, 2005). Usually, acute pancreatitis is relatively mild and resolves on its own, whereas chronic pancreatitis is more severe and is commonly defined as a continuing inflammatory process of the pancreas. Left untreated, chronic pancreatitis greatly increases the risk of developing pancreatic cancer (Raimondi et al., 2010). There are several different causes for pancreatitis, but all lead to a premature intra-pancreatic activation and release of digestive enzymes, resulting in cellular and tissue damage of the pancreas. As a consequence of the incomplete understanding of pathways and mechanisms involved in pancreatic diseases, therapeutic solutions remain inadequate (Wang et al., 2010).

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 enterokinases. Subsequently, trypsin activates the other pro-proteases needed for the digestion of proteins in the food. To

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doi:10.1016/j.ibmb.2011.04.012

prevent premature activation of the digestive enzymes, mammals express a protease inhibitor (PI) in their pancreatic cells called *pancreatic secretory trypsin inhibitor* (PSTI) (Hirota et al., 2006). The amino acid composition and sequence of bovine and human PSTI have been solved by conventional chemistry in the 1960s and 1970s (Greene and Giordano, 1969; Yamamoto et al., 1985). This inhibitor is a member of the Kazal-family and binds to prematurely activated trypsin, instantly interrupting the activation cascade. In patients that suffer from pancreatitis and/or pancreatic cancer, the activation cascade tends to start prematurely, resulting in severe damage to pancreatic tissues. In some cases the intra-pancreatic activation of digestive enzymes has been linked to mutations in the *PSTI* gene (Hirota et al., 2003; Witt et al., 2000). Also, knockout mice deficient in *PSTI* show autophagy-like symptoms in their pancreas and do not survive beyond 15 days (Ohmuraya et al., 2005). Interestingly, a serine protease inhibitor of the Kazal-family with a similar function has recently been identified in an evolutionary distant, cnidarian species, *i.e.* the freshwater polyp *Hydra magnipapillata* (Chera et al., 2006). This suggests an older, shared mechanism in protection against the potentially hazardous effects of digestive proteases. A better insight into this preserved mechanism across the animal kingdom may lead to a deeper understanding of the mechanisms of digestive enzyme regulation in general and of diseases like pancreatitis in particular.

In insects, several previous studies have shown the presence of Kazal-like inhibitors in – among others – *Bombyx mori* (Zheng et al., 2007), *Drosophila melanogaster* (Niimi et al., 1999), *Aedes aegyptii* (Ribeiro et al., 2007), *Triatoma infestans* (Lovato et al., 2006) and *Rhodnius prolixus* (Friedrich et al., 1993). Their biological and physiological functions are diverse, but so far a regulatory role on the activity of insect digestive proteases has not been discovered. In this study, a comparative *in silico* study of the available insect nucleotide sequence data revealed the presence of PSTI-like genes in Insecta (by far the largest class of animal species on planet Earth). Among many newly predicted PSTI-like genes a PSTI-homolog in the African migratory locust, *Locusta migratoria*, (LmPSTI) was identified and further characterized. LmPSTI was cloned, sequenced and its tissue distribution was investigated. Moreover, LmPSTI was produced using a bacterial expression system and its inhibitory effect on bovine and endogenous trypsin was confirmed *in vitro*. Knocking down this PSTI-like inhibitor via RNA-interference resulted in ultrastructural damage to the locust's caeca (the insect's functional equivalent of the mammalian exocrine pancreas) and established – for the first time in an insect – its functional role in a protective mechanism from prematurely activated digestive enzymes, remarkably similar to its role seen in mammals.

2. Methods

2.1. Rearing of animals

Gregarious migratory locusts, *L. migratoria*, were reared under crowded conditions with controlled temperature (32 ± 1 °C), light (14 h photoperiod) and relative humidity (40–60%). The animals were kept at high density (>200 locusts/cage) in special wooden cages and were fed daily with fresh grass and rolled oats. For all experiments in this study, freshly molted 5th larval stage animals were grouped on a daily base to exclude possible developmental differences.

2.2. cDNA cloning of LmPSTI

2.2.1. RNA extraction and cDNA synthesis

For the initial cloning and sequencing of *L. migratoria* PSTI (LmPSTI) and the subsequent tissue distribution analysis, total RNA from several tissues was extracted and reverse transcribed into cDNA. To obtain the total RNA, 5th larval stage animals were dissected and their tissues thoroughly washed in Ringer's solution. To maintain the integrity of the RNA, the tissues were immediately snap-frozen in liquid nitrogen and kept at −80 °C. Tissues were subsequently disrupted by means of the MagNA Lyser Instrument in 'Green Beads'-filled reaction tubes (Roche, Indianapolis, IN, USA). Total RNA was extracted with the Lipid Tissue RNA extraction kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. In combination with this extraction procedure, a DNase treatment (RNase-free DNase set, Qiagen) was performed to eliminate potential genomic DNA contamination. Synthesis of cDNA with SuperScriptIII Reverse Transcriptase (Invitrogen) was performed following the manufacturer's recommendations, using random primers and 1 μg of total RNA as starting material.

2.2.2. Cloning and sequence analysis

A putative LmPSTI sequence was derived from EST's retrieved by querying the NCBI database with the human (acc. nr. NP_003113) and mouse (acc. nr. NP_033284) PSTI protein sequences. Primers were designed to clone this sequence in a PCR reaction (Fw: 5′-CTCAATATGGACAGAAAGACCCT–3′; Rv: 5′-CAACCCGTTTCGCATATCTTAA-3′). Hot-start PCR was run for 30 cycles. 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 primer set and an extension step for 1 min at 68 °C, with a final extension step of 7 min at 68 °C. The PCR fragment was then subcloned using the TOPO-TA Cloning Kit for Sequencing (Invitrogen Life Technologies). After plasmid isolation (GenElute HP Plasmid Miniprep Kit: SIGMA), the insert was sequenced on a 3130 Genetic Analyzer (Applied Biosystems). A SignalP analysis (www.cbs.dtu.dk/services/SignalP) was performed to check for the presence of a secretion signal and the subcellular location of this inhibitor was predicted with TargetP (www.cbs.dtu.dk/services/TargetP).

2.3. In silico analysis

An extensive search in the public NCBI EST-database of insects revealed a multitude of putative Kazal-like inhibitors (*i.e.* the inhibitor family PSTI belongs to). Initial queries used in this search were the human PSTI and LmPSTI protein sequences, but these were progressively complemented with the newly-found insect Kazal-like PI sequences. Only the inhibitors which were expressed in the insects' digestive system were upheld and the amino acid sequences of those inhibitors were aligned with human PSTI using AlignX software (Invitrogen) (see Supplementary data 1).

2.4. Real-time RT-PCR transcript profiling

In order to study the tissue distribution of LmPSTI expression, several tissues were dissected from six animals: foregut (FG), midgut (MG), hindgut (HG), caeca (Ca), salivary glands (SG), brain (Br), gonads (Gn), fat body (FB) and malpighian tubules (MT). RNA extraction and cDNA synthesis was performed for each tissue separately as described above. Real-time PCR primers for the derived LmPSTI sequence as well as for the endogenous control (RP49) were designed with Primer Express software (Applied Biosystems). To verify the similar PCR-efficiencies of LmPSTI amplification reactions and the endogenous control, a validation experiment was performed. For this, standard curves were generated for the different transcripts with a serial (5×) dilution of a cDNA mixture of the different tissues. All reactions were run in duplicate on an Abi Prism 7000 Sequence Detection System (Abi Prism 7000 SDS, Applied Biosystems) using the following thermal