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Ph.D. Thesis

Peptidases and peptidase inhibitors in the salivary glands and the gut of *Nauphoeta cinerea*

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ANNOTATION

The present study is the first to report the identification, isolation, characterization of peptidase inhibitors from the salivary glands of *Nauphoeta cinerea.* The thesis consists of three articles. First publication presents data of the peptidase, amylase and peptidase inhibitor activities in the gut of six cockroach species. Second and third articles describe the purification, characterization, and expression of the five proteinase inhibitors from the alimentary canal of *Nauphoeta cinerea.*

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DECLARATION

I declare that I did all the work, presented in this thesis, on my own or in collaboration with the co-author of published articles, and with use of the cited references.

I declare that in accordance with the Czech legal code § 47b law No. 111/1998 in its valid version, I consent to the publication of my PhD. thesis (in an edition made by removing marked parts archived by the Faculty of Science) in an electronic way in the public access to the STAG database run by the University of South Bohemia in České Budějovice on its web pages.

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RESEACH OBJECTIVES

General goal of my work was verification of the occurrence of peptidase inhibitors in the digestive tract of phytophagous pest insects, with a remote perspective of their practical utilization. I used larvae of the Egyptian armyworm, EAW, (Spodoptera littoralis) and of the Colorado potato beetle, CPB, (Leptinotarsa decemlineata) as study objects. Using bovine α-chymotrypsin and trypsin as target peptidases, the only low inhibitory activity was found in both the wall and the lumen of EAW midgut. The attempts of inhibitors purification did not give a valuable result. Experiments with CPB indicated the presence some inhibitor activity in the midgut lumen, however it can't be excluded that this activity originated from the ingested potato leaves. Therefore, I have decided to use the cockroach *Nauphoeta cinerea* that was reported to contain high proteinase inhibitory activity in the gut (Elpidina et al., 2001a, b). The objective of my Ph.D. work was then biochemical and molecular biology characterization of these inhibitors.

In course of the work it was discovered that the inhibitors were derived from the salivary glands. Specific goals of my work were then defined:

1) Purification and characterization of peptidase inhibitors from the salivary glands of the cockroach *Nauphoeta cinerea* Oliv.;
2) Characterization of the cDNAs of these inhibitors and investigation of gene expression in different tissues and under different conditions;
3) Investigation of possible role of biogenic amines (serotonin and dopamine) in the regulation of gene expression and the secretion of peptidase inhibitors

This PhD thesis testifies that all goals have been reached. Five peptidase inhibitors were purified and characterized in respect to the peptidase substrates. Based on the N-termal sequence, full-length cDNAs of two inhibitors were amplified, cloned, and sequenced. Their putative translation identified two closely related Kazal-type inhibitors designated NcPIa and NcPIb. Both are active on subtilisin, NcPIa also on proteinase K and NcPIb on chymotrypsin. The corresponding genes are expressed specifically in the salivary glands. Their expression is not affected by starvation but it is stimulated by serotonin. Serotonin also stimulates and dopamine slightly suppresses the secretion of peptidase inhibitors from the salivary glands.

CHAPTER 1

Background information

INTRODUCTION

The insects use many different sources of food and exhibit an enormous diversity in the gut morphology and biochemical organization of digestion (Dow, 1986). The gut performs various physiological functions such as digestion of food (Teo and Woodring, 1989; Valaitis and Boweres, 1993; Valaitis, 1995), absorption of nutrients and electrolytes (Taylor, 1985; Dow and Harvey, 1988), secretion of waste materials, fluid transport, synthesis of hemolymph proteins (Palli and Locke, 1987), nourishment of hemocytes, conversion of ecdysone to 20-hydroxyecdysone, conjugation of ecdysteroids (Weirich et al., 1986; Weirich, 1997), and detoxification (Neal and Rueveni, 1992). The gut represents major interface between the insect and its environment (Law et al., 1992) and is commonly the attack site for invading microorganisms (Vallet-Gely at al., 2008). Immunohistochemical studies suggest that the gut is a rich source of biologically active compounds (Endo et al., 1982; Nishiitsutsuji- Uwo et al., 1985, 1986; Verhaert et al., 1986; Sehnal and Žitňan, 1990; Veenstra et al., 1995).

Although our knowledge of the physiology and biochemistry of insect gut is still rudimentary, a number of interesting observations suggest that this tissue deserves more attention for future investigation. I undertook a literature survey to review current understanding of the physiology of insect digestion, in particular in respect to proteolytic enzymes and their inhibitors both in the gut and in the salivary glands.

I. PROTEOLYSIS

Proteolysis is the breakdown of proteins into amino acids through the action of various proteases. Proteolysis plays important roles in all biological processes like blood clotting, tissue remodelling, nutrient acquisition, antigen processing and the mitotic cycle in all living organisms (Sternlicht and Werb, 2001). There are two forms of proteolysis: limited proteolysis and non-limited proteolysis (Thumm, 1993).

Limited proteolysis leads to the activation of immature proteins, inactive proteins, or zymogens by cleavage of one or a limited number of peptide bonds. Limited proteolysis occurs in proteins that undergo protein secretion, hormone processing and enzyme activation (Fuller et al., 1988). Limited proteolysis often acts through the action of proteases which act sequentially or at different levels of a cascade in a given biological phenomenon. For example, limited proteolysis can lead to the activation of one enzyme, which in turn activates a subsequent target in a cascade. Some of the classical examples of limited proteolysis are cascades controlling dorsoventral fate in *Drosophila melanogaster*,

hemolymph clotting in arthropods, complement and blood clotting in vertebrates (Bussey, 1988; Fuller et al., 1988; Krem and Di Cera, 2002).

Non-limited proteolysis is a process where the active biological molecules are rendered inactive through hydrolysis of many peptide bonds in the molecule. The liberated amino acids are either recycled back to protein synthesis or degraded in metabolic pathways to produce energy. Unlimited proteolysis inactivates active proteins upon receiving the intra or extra cellular signals. The proteins to be degraded are usually first conjugated with a small protein called ubiquitin. This modification makes them available for rapid ATP-dependent hydrolysis in proteasomes (large protein complexes containing degrading peptidases) abundantly presented in the cytoplasm of all cells. Another pathway includes proteases compartmentalized in lysosomes. Proteins transferred into this compartment undergo a rapid degradation by lysosomal cathepsins. Under various physical and biological conditions, the normal half life of protein varies from a few minutes to over 100 hours (Thumm, 1993).

Proteases perform many beneficial functions that are essential to life, but they are also dangerous and must be controlled. Proteolysis can be regulated by different mechanisms such as:

1) Specificity of proteases to their substrates;

2) Enzyme activation and inactivation;

3) Tagging;

4) Compartmentalization;

5) Inhibition.

2. PEPTIDASES

Proteolytic enzymes are essential for the survival of all kinds of organisms, and are encoded for by approximately 2% of all genes (Puente et al., 2005). Two main systems represent a comprehensive systematic classification of all known and putative types of proteases: the EC peptidase nomenclature (http://www.chem.qmul.ac.uk/iubmb/) and the peptidase database MEROPS (http://merops.iapc.bbsrc.ac.uk/). In both systems there are three major criteria currently used for the classification of peptidases: (i) the reaction catalysed, (ii) the chemical nature of the catalytic site, and (iii) the evolutionary relationship, as revealed by the structure.

According to the International Union of Biochemistry and Molecular Biology (IUBMB) nomenclature, proteases belong to the enzymes of group 3 (hydrolases), subgroup 4 (peptidases, act on the peptide bonds) (Int. Union Bioch., 1992). Peptide hydrolases (class 3.4), i.e. proteases can be further divided into families and clans. In reference to the site of cleavage of the peptide chain, we recognize exopeptidases and endopeptidases. Exopeptidases cleave peptide bonds at the amino terminus (aminopeptidase) or the carboxy terminus (carboxypeptidase). Endopeptidases, on the other hand, hydrolyze internal peptide bonds, away from either terminus of the protein substrate. Endopeptidases may be subdivided on the basis of the catalytic mechanism. Serine-type peptidases (EC 3.4.21) have a catalytic triad serine, histidine, aspartate (Ser, His, Asp) in the active site where serine residue acts as nucleophile atacking peptide bonds. The active site of cysteine peptidases (EC 3.4.22) contains a catalytic triad histidine, asparagin or aspartic acid and the nucleophilic cysteine residue (Cys, His, Asn/Asp).

Aspartic (EC 3.4.23) and metallopeptidases (EC 3.4.24) differ importantly from serine and cysteine peptidases in that the nucleophile that attacks the scissile peptide bond is an activated water molecule rather than the nucleophilic side chain of an amino acid. In aspartic peptidases, the water molecules that are involved in catalysis include aspartic residues that act directly as ligands for the activation water molecule. In the metallopeptidases one or two metal ions bind and activated water molecule.

There are also a number of peptidases for which the catalytic type remains to be established. They cannot be yet assigned to any of these sub-subclasses EC 3.4.21-24 and are listed as a separate sub-subclass (EC 3.4.99) of unclassified peptidases with regard to catalytic mechanism.

Barrett and coworkers have developed a classification scheme based on statistically significant similarities in the sequence and structure of all known proteolytic enzymes and constructed peptidase database MEROPS (Rawlings and Barrett, 1993; Barrett et., 2001). In that system all peptidases are assigned to families, and the families grouped in clans. The families of peptidases are constructed by comparisons of amino acid sequences. Every member of a MEROPS family shows a statistically significant relationship in amino acid sequence to at least one other member of the family. Moreover, it is required that the relationship exists in the part of the molecule named the “peptidase unit” that is most directly responsible for catalytic activity. Each family of

peptidases are assigned with a code letter denoting the type of catalysis, i.e., S, C, A, M, T or U for serine, cysteine, aspartic, metallo, threonine- or unknown type, respectively.

There are sets of families in which all of the proteins have diverged from a single ancestral protein, but they have diverged so far that their relationship can no longer be proved by comparison of the primary structures. The term "clan" is used in MEROPS classification to describe such groups of families. Usually the members of the clan have a distinct similarity in three-dimensional structure. The name of each clan is formed from the letter for the catalytic type of the peptidases it contains (as for families) followed by an arbitrary second capital letter. Presently there are 200 peptidase families grouped to about 40 clans (MEROPS release 6.9).

The active site of a peptidase is commonly located in a groove on the surface of the molecule between adjacent structural domains, and the substrate specificity is dictated by the properties of binding sites arranged along the groove on one or both sides of the catalytic site that is resposible for hydrolysis of peptide bond. Accordingly, the specificity of a peptidase is described by use of a conceptual model in which each specificity subsite is able to accommodate the side-chain of a single amino acid residue (Beynon and Bond, 2001). The sites are numbered from the catalytic site, S_1, S_2...S_n towards the N-terminus of the substrate, and S_1\`, $S_2$\`...S_n\` towards the C-terminus. The amino acids they accommodate are numbered $P_1$, $P_2$...$P_n$ and $P_1$\`, P_2\`...$P_n$\`, respectively, as follows:

Substrate: – P_3 – P_2 – P_1 + P1\`– $P_2$\`– P_3\` –

Enzyme: – S_3 – S_2 – S_1 * S1\`– $S_2$\`– S_3\` –

1.1. Serine peptidases

Over one third of all known proteolytic enzymes are serine peptidases grouped into 13 clans and 40 families (Di Cera, 2009). The family name is derived from the nucleophilic Ser in the enzyme active site, which attacks the carbonyl moiety of the substrate peptide bond to form an acyl-enzyme intermediate. Nucleophilicity of the catalytic Ser is typically dependent on a catalytic triad of Asp, His, and Ser residues, commonly referred to as the charge relay system. At least four distinct protein folds as illustrated by trypsin, subtilisin, prolyl oligopeptidase, and ClpP peptidase utilize the Asp-His-Ser catalytic triad in identical configuration to catalyze hydrolysis of peptide bonds. This is testimony to the success of the triad as a catalytic machinery in four distinct evolutionary pathways. Many serine peptidases employ a simpler dyad mechanism where Lys or His is paired with the catalytic Ser. Other serine peptidases mediate

catalysis via novel triplets of residues, such as a pair of His residues combined with the nucleophilic Ser. The substrate specificities of these enzymes are determined by their structures. In trypsin, for example, the active site serine is located next to a binding pocket that contains anionic aspartate, which attacks lysine and arginine of the substrate. The binding pocket of chymotrypsin is hydrophobic and interacts preferably with the phenylalanine, tyrosine, or tryptophan in the substate. Elastase`s type I binding pocket is shallow and binds small nonpolar residues, particulary alanine. Serine peptidases are usually endopeptidases and catalyze bond hydrolysis in the middle of a polypeptide chain. However, several families of exopeptidases have been described that remove one or more amino acids from the termini of target polypeptide chains. Within the metazoan lineage, a subset of serine peptidase families underwent significant gene duplication and divergence (Di Cera, 2009).

1.2. Insect serine peptidases

In many insects, serine peptidases are the major digestive and regulatory enzymes (Applebaum, 1985; Terra and Ferreira, 1994). Among them, trypsin- and chymotrypsin-like peptidases have important role in dietary protein digestion (Terra and Ferreira, 1994). They have been observed in midgut of various insect orders including Thysanura, Blattoptera, Orthoptera, Heteroptera, Coleoptera, Hymenoptera, Diptera, Lepidoptera (Gooding and Rolseth, 1976; Jany et al., 1978; Baker and Woo, 1981; Sakal et al., 1988; Ferreira and Terra, 1989; Baumann, 1990; Christeller et al., 1990; Graf et al., 1991; Johnston et al., 1991; Lemos and Terra, 1992; Zinlker and Polzer, 1992; Schumaker et al., 1993; Ferreira et al., 1994; Zhu et al., 2005; Volpicella et al., 2003; Zhu –Salzman et al., 2003; Vinokurov et al., 2006). In contrast to vertebrate trypsins, the insect trypsin-like enzymes are not activated by calcium ions (Levinski et al., 1977; Johnston et al., 1991; Lemos and Terra, 1992). Insect trypsins are unstable in acidic pH (Miller et al., 1974; Ward, 1975; Sakal et al., 1989) with the exception of some enzymes from *Musca domestica* and *Ostrinia nubilalis* (Lemos and Terra, 1992; Bernardi et al., 1996). Serine peptidases with elastase-like specificity were less studied in insects. Presence of elastase like enzymes has been documented in crickets (Christeller et al., 1989), caterpillars (Valaitis 1995; Milne and Kaplan, 1993) and *Tribolium castaneum* larvae (Vinokurov et al., 2009).

2.1. Cysteine peptidases

Peptidases in which the thiol group of a cysteine residue serves as the nucleophile in catalysis are defined as cysteine peptidases. Cysteine peptidases consist of one alpha-helix and four strands of antiparallel β-sheet. In cysteine peptidases discovered so far, the activity depends upon catalytic diad consisting of Cys/His. However, there are a number of peptidases with catalytic triad including Cys, His, Asn/Asp. The majority of cysteine peptidases are endopeptidases (papain, bromelain, ficain, cathepsins), but some act additionally or exclusively as exopeptidases (cathepsin X, carboxypeptidase B). Papain from the latex of *Carica papaya* represents an archetypal cysteine peptidase. Cysteine peptidases have been found in nearly all kinds of organisms, from the RNA and DNA viruses, eubacteria, protozoa, fungi, plants to the animals. The known cysteine peptidases have been classified into 35 sequence families combined to as minimum as 5 clans: CA (papain-like enzymes); CB (viral chymotrypsin-like CPs); CC (papain-like peptidases of RNA viruses); CD (legumain-type caspases) and CE (containing His, Glu/Asp, Gln, Cys residues in the catalytic cleft) (Barrett and Rawlings, 1996; Shearwin-Whyatt and Kumar, 2008).

2.2. Insect cysteine peptidases

In insects, cysteine peptidases were found to participate mainly in four major events, (1) the hydrolysis of yolk proteins during embryogenesis (Zhao et al., 2002, 2005), (2) proteolysis of dietary proteins in the digestive tract of larvae (Terra and Ferreira, 1994), (3) intracellular protein turnover in the lysosomes (Scott et al., 2004) and, (4) larval tissue histolysis during metamorphosis (Kawamura et al., 1984), in particular fat body dissociation during metamorphosis (Kurata et al. 1990; Xu and Kawasaki, 2001). Cysteine peptidases were also involved in *B. mori* silk gland histolysis (Shiba et al., 2001) and in the degradation of adult fat body during aging and oogenesis (Yang et al., 2006). Insects are unique because they use for digestion not only serine but also cystein cathepsin L- and cathepsin B-like peptidases secreted into the midgut lumen. The major insects groups with a cysteine type of digestion are bugs (Hemiptera) and Cucujiformia beetles (Coleoptera) (Johnson and Rabosky, 2000).

3.1. Aspartic peptidases

The aspartic peptidases comprise a family of around 100 members (www.merops.ac.uk/ merops/index.htm) including exclusively endopeptidases such as the vertebrate digestive pepsin, lysosomal cathepsin D, a kidney renin - angiotensin-

forming enzyme, and the secreted aspartic peptidases (Sap) produced by the human pathogen *Candida albicans* (Smolenski et al., 1997) and the plant pathogen *Glomerella cingulata* (Clark et al., 1997). Structurally, aspartic endopeptidases are bilobal enzymes, each lobe contributing a catalytic Asp residue, with an extended active site cleft localised between the two lobes of the molecule. One lobe has probably evolved from the other through a gene duplication event in the distant past. In modern-day enzymes, although the three-dimensional structures are very similar, the amino acid sequences are more divergent, except for the catalytic site motif, which is highly conserved. The presence and position of disulphide bridges are the other conserved features of aspartic peptidases.

3.2. Insect aspartic peptidases

The aspartate peptidases of insects are presented by cathepsin D (Terra and Ferreira, 1994.). The first report of aspartic peptidase in insects was made by Greenberg and Paretsky (1955), who found a strong proteolytic activity at pH 2.5-3 in the whole body homogenates of *Musca domestica.* The regulatory role in insect metamorphosis was described for cathepsin D (Gui et al., 2006). The same enzyme was described in the midgut of the blood-sucking Hemiptera as the secreted luminal peptidase (Houseman and Downe, 1983). Digestive aspartic peptidases were also described in the following families of Cucujiformia beetles (Meloidae, Chysomelidae, Coccinelidae) (Wolfson and Murdock, 1990) and Bruchidae (Silva and Xavier-Filho, 1991), but not in Tenebrionidae (Terra and Cristofoletti, 1996; Vinokurov et al., 2009). It cannot be excluded that aspartic peptidases become the digestive enzymes together with cysteine peptidases in the early evolution of Cucujiformia that specialized as a higher plants seeds and leaves feeders. The high abundance of protective molecules like serine peptidase inhibitors in the food consumed stimulated the shift from the initial serine to the mainly cysteine-aspartate type of digestion and then contributed to the success of this group of Coleoptera (Johnson and Rabosky, 2000).

4.1. Metallopeptidases

Metallopeptidases are the most diverse of the four main types of peptidases, with more than 50 families identified up to date. In these enzymes, a divalent cation, usually zinc, activates a water molecule. The metal ion is held in place by amino acid ligands, usually three in number. The known metal ligands are His, Glu, Asp or Lys and at least one

other residue is required for catalysis, which may play an electrophillic role. Of the known metalloproteases, around half contain an HEXXH motif, which has been shown in crystallographic studies to form part of the metal-binding site. The HEXXH motif is relatively common, but can be more stringently defined for metalloproteases as "abXHEbbHbc", where "a" is most often valine or threonine and forms part of the S_1' subsite in the thermolysin and neprilysin, "b" is an uncharged residue, and "c" a hydrophobic residue. Proline is never found in this site, possibly because it would break the helical structure adopted by this motif in metalloproteases (Beynon and Bond, 2001; Chang and Werb, 2001).

4.2. Insect metallopeptidases

A major digestive peptidase in the keratinolytic larvae of the webbing clothes moth, *Tineola bisselliella* (Lepidoptera: Tineidae), is a metallopeptidase. In spite of its occurrence and importance in *T. bisselliella* larvae, it is not a requisite for insect keratin digestion. The initial digestion in the keratinolytic larvae of *Attagenus megatoma* (Coleoptera: Dermestidae) is carried out only by the serine peptidases trypsin and chymotrypsin (Baker and Woo, 198l).

III. PEPTIDASE INHIBITORS

Peptidases perform many beneficial functions that are essential for life, but they are also dangerous and must be controlled. Several distinct mechanisms exist for the control of excessive peptidase activity, important amongst which are the interactions of the enzymes with proteins that inhibit them. Proteinaceous protease inhibitors (PIs) are molecules that form stable complexes with proteases and inactivate them.

PIs have been grouped into families and subfamilies and into different clans on the base of sequence relationships and the similarities of protein folds of the inhibitory domains or units. An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site and adjacent parts that are not directly involved in the inhibitor activity. Based on the sequence homologies of their inhibitor domains, PIs have been classified into 48 families (Rawlings et al., 2004). Proteins containing a single inhibitor unit are termed simple inhibitors, and those that contain multiple inhibitor units are termed complex inhibitors. A total of 11 families belong to the latter category and contain between 2 and 15 inhibitory domains. Most of these are homotypic, containing inhibitor units from a single family, some are however

heterotypic, and contain inhibitor units from different families (Richardson et al., 2001; Trexler et al., 2001, 2002). On the basis of tertiary structure, 31 of the 48 families have been assigned to 26 clans, indicating that a large proportion of families show no relationships in their three dimensional structures. The families of PIs could not, however, be grouped on the basis of the catalytic type of enzymes inhibited, since a number of families contain cross-class inhibitors (Rawlings et al., 2004). The proteins in family 13, the Kunitz-type PIs, generally inhibit serine peptidases, but they also include inhibitors of cysteine and aspartate proteases (Heibges et al., 2003). Family 14, the serpin family, contains mostly the inhibitors of serine proteases but also of some cysteine proteases. In the past, however, PIs have been classified into serine, cysteine, aspartate and metallocarboxy PIs (De Leo et al., 2002; Laskowski et al., 2003; Koiwa et al., 1997).

Protease inhibitors may be classified by their mechanism of action. Most protease inhibitors called “canonical”, mimic the protease substrate and thereby directly contact and block the active site of the enzyme. In other cases, the inhibitor does not bind directly to the substrate-binding site of the protease, but instead sterically prevents the uptake of the substrate (“non-canonical inhibitors”). Both above mentioned interactions are very tight, but reversible. Serpins have evolved a different, third extraordinary “mousetrap” mechanism of inhibition. Due to profound structural changes the serpin entraps the target protease in an irreversible complex (Huntington et al., 2000; Silverman et al., 2001). In all three cases, however, initial reversible interaction between the protease and the inhibitor in the active site of the protease or in adjacent position is a prerequisite for stable complex formation and inhibition (Bode and Huber, 2000; Huntington et al., 2000; Silverman et al., 2001). Elucidation of interactions between the protease and the inhibitor is essential for understanding the mechanism of inhibition and its successful application for the proteolysis control.

1. Serine peptidases inhibitors

Serine protease inhibitors make up the major group of protease inhibitors known and characterized so far. Serine protease inhibitors are classified in several families designated bovine pancreatic trypsin inhibitor (Kunitz) family, Kazal serine protease inhibitor family, soybean trypsin inhibitor (Kunitz) family, Bowman-Birk inhibitors (BBI) family, potato inhibitor family I and II, squash inhibitor family, barley trypsin inhibitor family, *Ascaris* trypsin inhibitor family, locust inhibitor family, ecotin and

serpin families, Streptomyces subtilisin inhibitor family, hirudin family, α-2-macroglobulin family.

The bovine pancreatic trypsin inhibitor (BPTI) family (Kunitz) of proteinase inhibitors is one of the most extensively studied protein families. Inhibitors of this family are small (50-65 amino acid residues) and possess six cysteines arranged in a characteristic disulphide bond pattern (Laskowski and Kato, 1980). The basic structure consists of N-terminal 3_{10}-helix around the first cysteine, a central double stranded anti-parallel β-sheet linked by a hairpin loop and a C-terminal three turn α-helix. The binding loop exhibits a characteristic conformation from P_3 to $P`_3$ and is stabilized by a cysteine at P_2 that is disulphide-connected to the hydrophobic core. The binding site-loop associates with the catalytic residues of the cognate enzyme in a similar manner as a productively bound substrate, with a P_1 carbonyl carbon fixed in contact with the reactive serine. The scissile peptide bond remains intact, with a slight out-of-plane deformation of the carbonyl oxygen. The P_3-$P`_3$ sites also interact with their cognate enzymes, while secondary contacts can also occur (Bode and Huber, 1992).

More than 100 **Kazal-type inhibitors** have been identified from vertebrates, arthropods, nematodes, and bacteria. Kazal-type inhibitors are mainly found in the blood plasma, pancreas, secretion of seminal vesicles and submandibular glands, as well as in the egg whites. The family of Kazal-type inhibitors shares several common structural features. These include a characteristic cystein distribution pattern, a typical VCGxD sequence motif and highly homologous three-dimensional structures. Crystallography and homology studies suggest that the amino acid residue located immediately after the second Cys residue in many Kazal inhibitors, corresponds to the reactive site and determines the specificity of the inhibitor (Bode and Huber, 1992). Different Kazal inhibitors may arise from alternative splicing of the same gene, for example alternate splicing of KAZ1 results in several Kazal-type SPIs in *Drosophila melanogaster* (Niimi et al., 1999).

The serpins on the other hand constitute a family of large (glyco)proteins (typically about 400 amino acid residues in length) which function as suicide substrate inhibitors. Upon binding, they are cleaved by the target protease within a reactive center loop region of about 20 amino acids near the C-terminus. The amino acid in P1 position appears to be important for determining the specificity of serpins for particular proteases. Further N-terminally within the reactive center loop region are the residues of the hinge region that are highly conserved among inhibitory serpins. Serpins are

involved in diverse biological processes. In insect hemolymph serpins block the activity of serine peptidases linked with prophenoloxidase activation, hemolymph coagulation system and exogenous peptidases of microbial and fungal pathogens (Kanost, 1999).

Hirudin is a potent thrombin inhibitor secreted by the salivary glands of *Hirudinaria manillensis* (Buffalo leech) and *Hirudo medicinalis* (Medicinal leech). It forms a stable non-covalent complexes with peptidases involved in coagulation cascade (thrombin and factor XA). Hirudin consists of an N-terminal globular domain and an extended C-terminal domain. The C-terminal functional domain of hirudin binds to a non-catalytic site on thrombin.

The distinguishing feature of **α-macroglobulin family** is the presence of a "trap" with cyclic thioether on the bottom and of a sufficiently large hydrophobic area. Inhibitors of the α-macroglobulin family have been proven to occur in molluscs, fish, amphibians, reptiles, ticks, insects, birds, and mammals. Human α-2-macroglobulin has been accepted as a classical member of this family (Zorin et al., 2006).

Recently, two new families of low molecular weight serine protease inhibitors have been discovered. The first, designated as the ***Bombyx* family** was discovered in the silkworm. The second was designated **locust serine protease inhibitor peptide family** (Schoofs et al., 2002).

2. Cysteine peptidases inhibitors

Within the last ten years it has become evident that the newly discovered protein inhibitors of cysteine peptidases, named cystatins, offer a new insight into the processes in which the inhibitors participate. These inhibitors might protect the cells from inappropriate endogenous or external proteolysis and/or could be involved in the control mechanism responsible for the intracellular or extracellular protein degradation. The cystatins are tightly and reversibly binding inhibitors of the papain-like cysteine proteinases. They form a superfamily of sequentially homologous proteins subdivided into four families: stefins, cystatins, kininogens, and phytocystatins (cathelin).

The stefins (also called family I cystatins) are small, acidic proteins, consisting of about 100 amino acid residues and lacking disulphide bonds. The best characterized representatives are the human stefins A and B. **The cystatins** (Family II cystatins) are somewhat larger proteins than the stefins, consisting of about 115 amino acid residues and having two disulphide bridges. The name "**cystatin"** was first used by J. Barrett in 1981 to describe a protein that had been discovered and partially characterized from the

chicken egg-white as an inhibitor of papain, ficin and other related cysteine endopeptidases. Cystatins occur at relatively high concentrations in many biological fluids such as human seminal plasma, cerebrospinal fluid, plasma, saliva and urine. Both the stefins and the cystatins are competitive, reversible inhibitors which form tight, equimolar complexes with their target proteinases. Three regions of the inhibitors were shown to interact with the enzyme: two hairpin loops and the N-terminal part, which appears to be more important in the cystatins.

Kininogens have long been known as precursor proteins of the vasoactive kinins and as participants in the blood coagulation cascade. There are three distinct types of kininogens, designated as high molecular weight kininogen (H-kininogen) with Mr of about 120 000 Da, low molecular weight kininogen (L-kininogen) with Mr of about 68 000 Da, and T-kininogen (also known as "major acute phase protein") with Mr of about 68 000 Da. They are all single-chain proteins (Turk and Bode, 1991).

Cysteine peptidase inhibitors may be involved in the control of endogenous cysteine peptidases and may also play a protective role against invasive organisms that use cysteine peptidases for penetration (Turk et al., 1995).

3. Aspartic peptidases inhibitors

Proteinaceous inhibitors of aspartic peptidases are relatively uncommon and are found in only a few specialized locations (Bennett et al., 2000). They include renin-binding protein present in mammalian kidney, now specified as *N*-acetyl-D-glucosamine-2-epimerase (Kay et al., 1983; Phylip et al., 2001), a 17-kDa inhibitor of pepsin and cathepsin E from the parasite *Ascaris lumbicoides* (Kageyama, 1998; Ng et al., 2000), proteins from plants such as potato, tomato, and squash (Kreft et al., 1997; Christeller et al., 1998), and a polyfunctional inhibitor of cathepsin D from the sea anemone acting on cysteine peptidases as well as on cathepsin D (Lenarcic and Turk, 1999).

4. Inhibitors of metallopeptidases

Tissue inhibitors of metalloproteinases (TIMPs) are the natural inhibitors of matrix metalloproteinases (MMPs) found in most tissues and body fluids. Four TIMPs (TIMP-1, -2, -3 and -4) have been identified. Like MMPs, the expression of TIMPs in the tissue is also controlled to maintain a balance in the metabolism of the extracellular matrix. Disruption of this balance may result in a number of pathogenic processes. TIMP-1, TIMP-2 and TIMP-4 are present in soluble forms, while TIMP-3 is tightly bound to the

matrix. Numerous studies have indicated that TIMPs are multifunctional proteins involved not only in tissue remodeling and wound healing but also in many other physiological and pathological processes such as angiogenesis, steroidogenesis, hematopoiesis, cell growth and cell survival (Baker et al., 2002).

5. Insect peptidases inhibitors

Insect's hemolymph is a very rich source of peptidase inhibitors. There were identified inhibitors from the Kazal, Kunitz, α-macroglobulin and serpin families (Eguchi, 1993; Polanowski and Wilusz, 1996) playing essential role in the regulation of proteolytic-activated processes, such as the phenoloxidase cascade, as well as in metamorphosis, development, and defense against invading pathogens (Ramesh et al., 1988; Sugumaran et al., 1985). Thus, they can be used as powerful tools for elucidating insect immunity at the molecular level (Kanost, 1999).

The prophenoloxidase activating system is considered to be responsible for defensive functions in insects as well as in other invertebrates (Pye, 1978; Ratcliffe et al., 1984; Leonard et al., 1985; Soderchall and Smith, 1986; Yoshida and Ashida, 1986; Ashida and Yoshida, 1988). Endogenous serine peptidase inhibitors in the hemolymph of *Manduca sexta* and *Sarcophaga bullata* were found to prevent activation of prophenoloxidase by inhibiting the activating protease (Sugumaran et al., 1985). Another important role of inhibitors could be protection against invading pathogens and parasites that use peptidases to penetrate through the integument (Eguchi, 1982). It is noticeable that inhibitory activity against fungal peptidases was found in silkworm integument that represents the first barrier for the invading fungi (Yoshida et al., 1990).
The strong inhibitory activity of silkworm hemolymph against endogenous proteolytic enzymes may protect tissues from the deleterious effects of proteases which may leak into the hemolymph from the alimentary canal damaged by bacteria or viruses (Eguchi, 1982). In connection with this problem, the marked developmental change in the hemolymph inhibitor with its highest activity in the spinning period implies that the one role might be inhibition of peptidases discharged from various tissues during histolysis (Eguchi et al*.,* 1986). Indeed, changes in the hemolymph inhibitor activity show good correlation with the progress of metamorphosis (Eguchi and Kanbe, 1982; Eguchi et al*.,* 1986). Suzuki and Natori (1986) reported that the amount of cysteine peptidase inhibitor, sarcocystatin A, from the flesh fly *Sarcophaga peregrina* reached a peak in

the middle pupal stage, consistently with extensive disintegration of larval tissues at this time.

The serine peptidase inhibitors from hemolymph where thoroughly studied in many insects such as *Bombyx mori* (Eguchi et al., 1982; Sasaki and Kobayashi, 1984; Kurata et al., 2001), *Antheraea pernyii* (Eguchi et al., 1982), *Drosophila melanogaster* (Kang and Fuschs, 1980), *Manduca sexta* (Kanost et al., 1989; Kanost, 1990; Ramesh et al., 1988; Sugumaran et al., 1985; Gan et al., 2001), *Locusta migratoria* (Boigegrain et al., 1992; Simonet et al., 2002), *Galleria mellonella* (Frobius et al., 2000), *Schistocerca gregaria* (Hamdaoui et al., 1998; Gaspari et al., 2002), *Theromyzon tessulatum* (Chopin et al., 2000), *Mythimna unipuncta* (Cherqui et al., 2001), and *Antheraea mylitta* (Shrivastava and Ghosh, 2003). Activities of trypsin-like proteases and their inhibitors in the hemolymph, midgut, and fat body were examined in *Galleria mellonella* caterpillars in relation to developmental changes and several types of stress (Kučera et al., 1984).

Serine peptidase inhibitors were also found in the salivary secretion. Their functions are clearly elucidated only in hematophagous insects where they act as anticoagulants directed against serine peptidases of coagulation cascade - factor IIa (thrombin) and factor Xa (Ribeiro, 1995). Anticoagulant factor Xa from the serpin superfamily of serine peptidase inhibitors was purified and characterized from the salivary glands of the mosquito *Aedes aegypti* (Stark and James, 1995). A novel non-homologous highly specific anti-thrombin inhibitor anophelin was identified in the salivary glands of the closely related species *Anopheles albimanus*. It has no cysteines, which is similar to the anticoagulant peptides of the leeches and ticks (Valenzuela et al., 1999). The modern approaches of transcriptome analysis revealed the presence of Kunitz and Kazal-type peptidase inhibitor specific transcrips in the salivary glands of black flies (*Simulium vittatum*) (Andersen et al., 2009) and mosquitoes (*Anopheles darlingi*) (Calvo et al., 2004). Labial silk glands of the waxmoth larvae *Galleria mellonella* and the silkworm *Bombyx mori* produce low molecular weight Kunitz- and Kazal-type peptidase inhibitors highly active against trypsin and microbial peptidases – subtilisin and proteinase K. Their genes are expressed in the middle part of the gland where inhibitors become associated with sericins involved in the coccoon shell formation (Kurioka et al., 1999; Nirmala et al., 2001a, b). As it was proposed these inhibitors can protect the silk proteins from degradation by endogenous peptidases (Kurioka et al., 1999) or from exogenous fungal or microbial enzymes (Sehnal and Sutherland, 2008). Peptidase

inhibitors cDNAs were also identified in the salivary glands of the hessian fly larvae (*Mayetiola destructor)*. Based on the analysis of deduced amino acid sequence of these proteins it was concluded that they belong to the *Ascaris* family of peptidase inhibitors (Maddur et al., 2006). These proteins, active against commercial trypsin and chymotrypsin, were highly expressed during the period of active larval feeding (1st and 2nd instar). They are components of the hessian fly salivary gland secretion that is injected into the plant tissue and suppress activation of the plant resistance mechanisms. However, the direct peptidase inhibitors role in these processes has not been investigated (Chen, 2008). Peptidase inhibitors with unknown functions were found in the midgut of several cockroach species belonging to the family Blaberidae, *Leucophaea maderae* (Engelmann and Geraerts, 1980), *Nauphoeta cinerea* (Zhuzhikov, 1997; Elpidina et al., 2001b; Vinokurov et al., 2007) and *Blaptica dubia* (Vinokurov et al., 2007). So, regardless of the sufficient amount of literature about the insect peptidase inhibitors the most of them are still proteins in search of a function rather than proteins isolated to account for a previously discovered biological function.

IV. ORGANISATION OF DIGESTION IN THE COCKROACHES

1. Morphology of the alimentary canal

Most cockroaches are omnivorous and feed on different kinds of organic matter. Their digestive tract is a tube composed of several compartments specialized for: food intake, grinding, and storage, digestion and nutrient resorption, water resorption and feces formation.

Digestive system includes the mouth parts, a pair of salivary glands and the alimentary canal. The mouth parts (mandibles, maxillae, and labium) of cockroaches are adapted to biting and chewing food. Food is taken into pharynx and continues to short, narrow and thin-walled oesophagus. The three divisions of the cockroach alimentary canal are: the foregut, which includes the crop and proventriculus, midgut with a different amount of caeca on the anterior end and the hindgut including ileum and rectum. In cockroaches proventriculus is a grinding apparatus with strong cuticular plates breaking up the solid food. The opening of the proventricular valve, which allows transport of ground food (chylus) to the midgut, was examined in the cockroach *Leucophaea maderae* and found to be controlled by the stomatogastric nervous system (Engelmann, 1968).

The products of digestion are absorbed in the midgut, especially in its anterior part containing gastric caeca. They increase the surface area for nutrient absorption and are

also the site of digestion and production of the enzymes. In the german cockroach *Blattella germanica* the number of gastric caecae is 10 but may vary from 4 to 12 depending on the physiological status (Rust et al., 1995). Absorption also occurs in the hindgut, especially in the rectum, but there is no evidence of absorption from the foregut, which has an impermeable cuticular lining.

Hui Ma (2009) adopted a three-dimensional (3-D) reconstruction technology to generate a 3-D model of the digestive system of the larval *Periplaneta americana.* His observations showed that the digestive system occupies a large part of the body cavity. The tubular alimentary canal from mouth to anus is twice as long as the body. The foregut comprises almost one half of the digestive system (43.57%). The midgut, the critical region for digestion and absorption, has the second highest volume ratio (35.21%). The hindgut has the lowest volume ratio (21.22%).

2. Structure and function of the salivary glands

The cockroaches has a typical paired acinous type salivary glands lying on either side of the foregut and accompanied by distensible sacs called reservoirs. The fluid-secreting regions or acini lie on the terminal end of the branching system of the salivary ducts and are usually composed of the several cell types (Kendall, 1969; House and Ginsborg, 1985; Just and Walz, 1994). The acini produce and secrete saliva flowing through the salivary ducts system into the pre-oral cavity (House and Ginsborg, 1985). Salivary secretions moist the food and contains digestive enzymes starting digestion in oesophagus and crop (Gardiner, 1972; Kendall, 1969).

The four main cell types with different functions were found in the salivary glands of cockroaches. The acini are composed from the central cells (C-cells) and the peripheral cells (P-cells). The central cells contain rough endoplasmatic reticulum, Golgi bodies and a large granules (*ca.* 2 mkm diameter) with no prominent membrane foldings. Peripheral cells are pyramidal in shape with a plenty of mitochondria in cytoplasm providing energy for the active ion transport performed by a Na+/K+-ATPase located in the numerous apical membrane foldings of these cells (Just and Walz, 1994) (Fig.1A).
Ginsborg et al. (1980) and Rietdorf et al. (2005) suggested that C-cells secrete proteins, whereas P-cells transfer ions and water into the acinar lumen forming the fluid component of the saliva. The central cells secrete amylase and probably also the other enzymes (invertase, maltase, and protease) found in the glands of *Nauphoeta cinerea*. Lactase and lipase were not detected in the gland although the latter enzyme was present

in the gut. Acinar cells are joined by septate desmosomes and gap junction (House, 1980).

In the vicinity of the acini lie so called secretory duct cells containing dense granules in cytoplasm and producing the mucous component of saliva (sialoglycans). More distantly from the acini the non-secretory duct cells participating in reabsorption of ions from the primary saliva are located. These cells have prominent basal membrane infoldings with a numerous mitochondria arranged between them providing energy for the active ions re-adsorption into hemolymph (House, 1980).

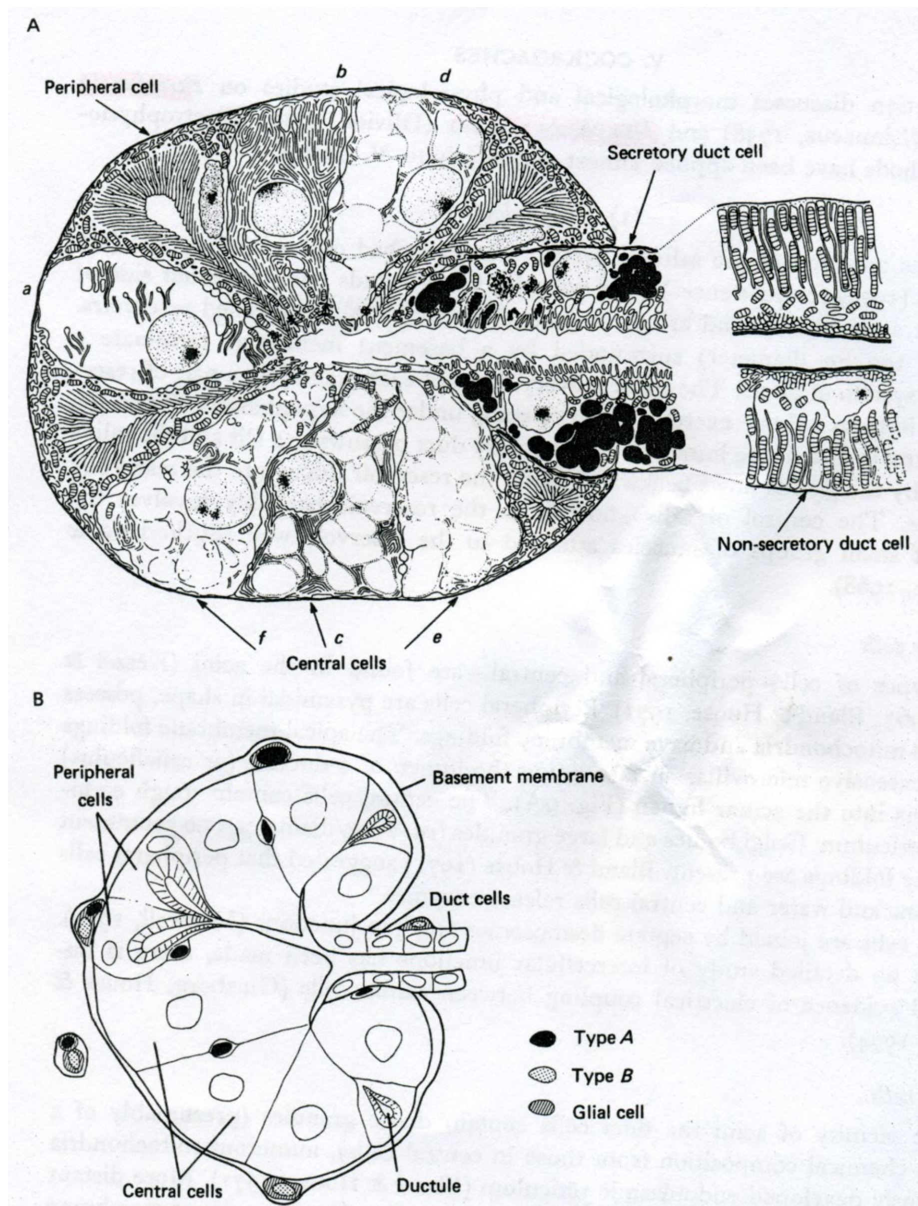

Fig. 1. Diagram of an acinus from the salivary gland of *Nauphoeta cinerea* (House, 1980). A, four cell types are shown. The sequence *a-f* shows central cells in different stages of activity: *a*, completely depleted cell with regeneration of endoplasmatic reticulum just beginning; *b*, regeneration of endoplasmic reticulum complete; *c*, production of granules underway; *d*, and *e* cells with granules; *f*, following the release of granules, the residual empty structures disappear to give

rise to a cell like that shown in *a*. B, the acinar positions of the salivary nerve terminals of type *A* and *B*.

Salivary glands secretory activity is controlled by serotonergic and dopaminergic neurons whose axons form a dense plexus on the glands. Immunofluorescent labeling of nerve fibres with anti-dopamine and anti-serotonin showed that acini are entangled in a dense plexus of dopaminergic and serotonergic fibres. The former reside on the surface of the acini next to P-cells, whereas the latter invade the acini and form a dense meshwork between C-cells. Duct segments close to the acini are locally associated with both dopaminergic and serotonergic fibres, whereas the duct segments further downstream have only dopaminergic innervation. Serotonin stimulate C-cells that results in the production of proteinaceous components of the saliva, whereas dopamine acting on the P-cells induces the production of protein-free saliva and consequently responsible for the secretion of its fluid components (Just and Walz, 1996).

3. Nutrient requirements

The principles of the major insect nutritional requirements for growth and reproduction have been established since 1940's through the studies on representatives of the major insect groups (Genc, 2006). Nutrition is described as chemicals required by an organism for its growth, tissue maintenance, reproduction, and energy. Most of these chemicals are taken during feeding, some are synthesized by the insects (Chapman, 1998).

Insects may respond to imbalanced diet in three ways. They can alter the total amount of ingested food; they can move from one food to another with a different nutrient balance; or they can regulate the effectiveness of the nutrient uptake. The required balance of the nutrients such as carbohydrates, proteins, lipids, vitamins and amino acids, is generally related with the natural foods of the species (Dadd, 1985). Predatory insects have high amino acid requirements relative to the carbohydrates, reflecting the protein content of animal tissues (Chapman, 1998). Phytophagous insects such as Orthoptera, Coleoptera, and Lepidoptera generally require almost equal amounts of proteins, amino acids and carbohydrates. Phloem-feeding insects and the grain beetles have high requirements for carbohydrates. The nutritional requirements can change with time, depending on the growth, reproduction, diapause or migration. It is usually true that the nitrogen content of insect larvae is higher in the early instars, while lipids are

accumulated in the later instars as reserves for metamorphosis and reproduction (Nation, 2001; Chapman, 1998).

Most insects need an optimal level of proteins in their diet. They are hydrolyzed to amino acids that are largely used for the synthesis of endogenous structural proteins, enzymes, receptors, etc. (Chapman, 1998). Proteins are crucial for the ovary and egg development. Male insect usually do not require protein intake to mature their sperm when they become adults. Haydak (1953) showed that restricted uptake of dietary proteins in several cockroaches slowed down their growth, but prolonged longevity. The American cockroaches grew fastest on diets containing 49% and 78% protein but survived longest on those with 22% to 24 % protein.

Carbohydrates are very important energy source but they are not essential diet components because they can be synthesized from the lipids or amino acids (Nation, 2001). Different carbohydrate utilization depends on the ability of insects to hydrolyse polysaccharides. Most insects are unable to use cellulose and other plant polymers because they do not have the enzymes to digest them. In some insect species, these food components are digested by symbiotic microorganisms (Nation, 2001, Chapman, 1998). Insects are able to convert carbohydrates into lipids that are usually accumulated in the fat body. All insects are believed to require dietary steroids because they cannot synthesize the sterol rings. Besides being a part of all cellular membranes, sterols are precursors for the synthesis of the ecdysteroid molting hormones. Thus, deficiency of sterols in the diet results in incapability of the insects to molt and they typically die in an early instar (Nation, 2001; Genc, 2002).

Insects usually require a good source of seven vitamins (thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, folic acid, and biotin) that cannot be synthesized by the insects or their symbionts. The mineral requirements of insects are inadequately known. Given the known composition of insects, it is reasonable to assume that sodium, potassium, calcium, magnesium; chloride and phosphate are essential (Nation, 2001). Several metal ions are essential as enzyme co-factors; for example, molybdenum is a part of the xanthine dehydrogenase that is important in purine metabolism.

Cockroaches are of particular interest in the study of nutritional regulation, owing to the fact that they are extremely opportunistic scavengers (Schal et al., 1984) and the diversity of substances that they use as foods is greater than in any other insect order (Cornwell, 1968). For this reason, cockroaches are less able than any other group of insects to rely upon the nutritional composition of their foods as source of a nutritionally

adequate diet. Therefore, they might be expected to have evolved efficient and accurate means of endogenously regulating their nutrient status. Cockroach biology in general, and nutrition in particular, has been studied consistently for a long period (e.g. for the German cockroach, *Blattella germanica*: Hummel, 1821; Sanford, 1918; Abbott, 1926; Melampy and Maynard, 1937; Noland and Baumann, 1951; Gordon, 1968; Cochran, 1983; Cooper and Schal, 1992), on account of the ease of cockroach rearing (Scharrer, 1951) and their economic importance as pests (Cornwell, 1968). Considerable progress has been made through such studies, which have revealed some unusual and important nutritional adaptations in cockroaches. For instance, most insects store excess carbohydrate as fat for future metabolic use, but either void excess nitrogen to the exterior or store it in protein form (Telfer and Kunkel, 1991).

Cockroaches, by contrast, evolved the ability to accumulate the excess of nitrogen in the form of urate salts in the fat body than excreting them (Cochran, 1985). Stored urates can be recycled by bacterial endosymbionts metabolising urates into utilizable nitrogenous compounds released into hemolymph in the case of nitrogen deficiency (Valovage and Brooks, 1979). Further, numerous studies revealed the essential nutrients for cockroach growth and reproduction (Haydak, 1953; Gordon, 1959; Cochran, 1983; Durbin and Cochran, 1985; Silverman, 1986; Hamilton and Schal, 1988; Cooper and Schal, 1992). It was established that adult cockroaches prefer diets with a high carbohydrate level over a protein rich diets. However, the rates of protein and carbohydrate consumption changes significantly depending on the instar and physiological status. In the experiments with cockroach nymphs the weight gain and percent of survival from the hatching to the adult stage was maximal, if the cockroaches were able to select combination both of these components according with their needs (self selection) (Ross and Mullins, 1995; Jones and Raubenheimer, 2001).

4. Digestion in the gut lumen

A large part of the food ingested by insects is macromolecular, in the form of polysaccharides and proteins, while lipids are present as triglycerides, phospholipids and glycolipids. Generally all large food polymers must be digested into the small molecules like amino acid short peptide molecules and mono- and disaccharides that may be absorbed by midgut epithelium. Digestive enzymes are present in the saliva and in the midgut secretion. In addition, digestion may be performed by enzymes derived

from the gut of the microorganisms. Food passes through the proventriculus into the midgut where it is separated from the gut epithelium by the peritrophic matrix (PM) – the complex membranous structure formed by chitin fibers embedded into the complex protein-mucopolysaccharide matrix. The PM of cockroaches is secreted by the cells located at the beginning of the midgut at the base of the gastric caecae (cardia) and also by the entire midgut digestive epithelium (the combined type). The several functions are attributed now to PM:

1. it may serve as a barrier to microbial infection or abrasion by food particles;
2. a molecular sieve regulating the longitudinal and cross-midgut translocation of enzymes and food molecules (Terra, 1990).

Based on the physiology, it is possible that all insects have the full complement of ordinary digestive enzymes, the relative amounts of which change in response to diet composition. This change may occur during the feeding of one individual (Applebaum, 1985), or may be the result of the adaptation of a taxonomic group of insects to a particular diet (Terra and Ferreira, 1994). Furthermore, the compartmentalization of digestive enzymes has drawn attention to the fact that the overall pattern of digestion, and the gross and ultrastructural morphology of the midgut, correlate well with the phylogenetic position of the insect (Terra 1988; 1990).

5. Enzymology of gut

a. Regulation of enzyme activity

There are three main cell types we distinguish in the insect midgut: columnar cells, regenerative cells and endocrine cells.

The main sources of the insect digestive enzymes are the columnar cells – predominant cells in the insect midgut. They are generally involved in water and nutrients absorption, water secretion, digestive enzyme synthesis and secretion. The highly folded apical plasma membrane forms the numerous microvilli containing glycocalyx bounded digestive enzymes participating in the final stages of midgut digestion. It is so called membrane digestion leading to the formation of the high monomers concentration in the vicinity of the adsorbtion sites. The second cell type of midgut epitheliums are the small regenerative cells often clumped in small clusters at the base of the columnar cells. The third type of midgut cells are the endocrine cells distributed throughout the epithelium and containing in cytoplasm electron-dense granules. Recently, gene expression of insect neuropeptides such as allatostatin,

leucomyosuppressin, neuropeptide F and CCAP have been detected in these cells (Chapman, 1998; Sehnal and Žitňan, 1996). In the highly alkaline midgut of Lepidoptera larvae and the acidic midgut of the larvae of Diptera Cyclorrhapha there are two additional specialized cells: goblet cells found in Lepidoptera and responsible for the active transport of potassium ions from hemolymph to the midgut lumen (Harvey et al., 1983) and oxyntic cells in Diptera that are thought to support the low midgut acidic pH (around 3.0) by pumping protons into the midgut lumen (Terra, 1988).

The stomatogastric nervous system innervates only midgut musculature, indicating that digestive and absorptive cells in the midgut epithelium are not regulated by nerve tissue. Thus, nonneural regulatory mechanisms based on the peptides hormones produced by midgut endocrine cells are expected to regulate digestion and absorption in insect midgut (Lehane et al., 1996). Accordingly, it was found that trypsin and amylase secretion could be induced in isolated midguts of mosquitoes, *Stomoxys calcitrans* and cockroaches after it incubation in the media with some peptide hormones (Lehane et al., 1995; Sakai et al., 2004; 2006). It is well documented that the ingestion of some nutrients will stimulate synthesis of digestive enzymes (so called secretagogue control mechanism). There is evidence that such stimulation depends on the concentration of ingested nutrient (not on the size of the meal) and the type of food consumed. In cockroaches only ingestion of proteins stimulated peptidase activity in the gut. Similarly, the rise of amylase was stimulated by the ingested starch, however the consumption of non-nutritional cellulose did not influence the enzyme secretion level (Engelmann, 1969; Lehane et al., 1996). It was shown that probably peptide hormones like CCAP produced in the midgut endocrine cells act as mediators of enzyme secretion. Nutrients like casein or starch stimulate the production and release of hormones, which in turn up-regulates peptidase and amylase activity (Sakai et al., 2006). Hovewer, the detail knowledge of insect digestive enzyme regulation is still under the study and midgut hormone receptors have not yet been identified.

b. pH and the redox potential

According to Johnson and Felton (1996), three factors remarkably affect insect digestion: pH, buffering capacity, and the redox potential.

The pH generally increases from foregut to midgut and then decreases to the hindgut. This pattern is true for most phytophagous species but in many omnivores and carnivores the pH of the hindgut is greater than that of the midgut. Generally, pH of the

crop content is the same as that of the food. In some species the pH is consistently less that 7, because of the digestive activity of microorganisms or because of the regurgitation of digestive juice from the midgut. The pH of the midgut differs among species but tends to be constant for a given species because of the presence of buffering agents. The hindgut typically has a pH slightly less then 7, presumably resulting from the presence of nitrogenous waste product, the uric acid, or as a result of the formation of organic acids from the cellulose hydrolyzed by symbiotic microorganisms. Results of our pH measurements in cockroach gut compartments indicated similarities between species of the same family. pH varies along the gut length from alkaline to acidic in *B. orientalis* and from acidic to alkaline in both Blaberidae species as reported by Vinokurov et al. (2007).

The redox potential reflects ability to gain or lose electrons, i.e., to be reduced or oxidised, respectively. Normally, redox potential of the gut is positive, which is indicative of oxidizing (aerobic) condition. However, in species able to digest keratin the redox potential of the midgut fluid is strongly negative. It has been suggested that such anaerobic (reducing) environment is necessary for splitting of disulfide bridges (reduction of S^0 to S^{-1}) between polypeptide chains of keratin molecules (House, 1974). In the wood feeding cockroaches and termites, the negative redox potential found in the hindgut fermentation chambers is related with the presence of anaerobic cellulose degrading microbes (Bignell, 1984a, b; Veviers et al., 1982). The redox potential of the gut is largely dependent on pH and the redox activity of ingested material.

Inter- and intraspecific variation in midgut pH and redox conditions appears to be important to influence digestion via effects on the structure and function of dietary proteins and proteolytic enzymes. Gut pH among the herbivorous insects tends to be correlated with their phylogenetic position, while redox conditions are more variable and often reflecting the current adaptations to the specific diet or digestion type.

Recent investigations in herbivorous insects revealed that the broad range of gut redox and pH conditions ranging from -200 mV to +260 mV and from pH 6.0 to pH 11.8 (Johnson and Felton, 1996).

c. Compartmentalization of digestive enzymes

Organization of the digestive process depends on compartmentalization of secreted enzymes and on midgut fluid stream that are responsible for the translocation of enzymes and products of digestion (Terra and Ferreira, 1994). The digestive processes

occur in three phases: initial, intermediate and final. Initially, a decrease in molecular weight of polymeric food molecules occurs through the action of polymer hydrolases, such as amylases, cellulases, and endopeptidases. The resulting oligomers then undergo hydrolysis by polymer hydrolases (e.g. amylase) or are hydrolyzed by oligomer hydrolases, exemplified by aminopeptidase acting upon protein fragments. The products of this phase are dimers or small oligomers such as maltose, cellobiose, and dipeptides. During final digestion, dimers are split into monomers by dimer hydrolases, such as maltase, cellobiase and dipeptidase. The compartmentalization of the digestive enzymes has been studied in numerous insects. The first attempt to relate midgut compartments to each of these phases of digestion, and hence to the corresponding enzymes, was accomplished by Terra et al. (1979) in the larvae of *Rhynchosciara americana* (Diptera: Sciaridae).

The enzymes involved in initial digestion are either in the foregut or in the endoperitrophic space of the midgut surrounded by PM, whereas those participating in the intermediate or final digestion localized to the ectoperitrophic space (between PM and midgut epithelium) or associated with the cell glycocalyx (microvillar enzymes) (Terra, 1988; 1990).

d. Digestion of carbohydrates; amylase activity of the digestive tube

Starch and glycogen are digested by amylases and common disaccharides sucrose and maltose by α-glucosidases. The naturally occurring β-glucosides are usually of plant origin and the highest β-glucosides activity is found in phytophagous insects. Cellobiose is a product of cellulose digestion and a cellobiase is often present even in insects where cellulose digestion is not known to occur (Chapman, 1998). Utilization of the most abundant structural polysaccharides in nature, the cellulose and chitin, is limited and often dependent on the symbiotic microorganisms (Vonk and Western, 1984). However, cockroaches produce endogenous cellulases in addition to those secreted by their symbionts (Slaytor, 1992).

The salivary glands of cockroaches secrete amylases and in *Blatella germanica* (though not in *Periplaneta americana*) also an invertase. In the midgut of *P. americana* amylase activity was localised in the two regions: in the caecal epithelium and anterior ventricular epithelium. In both regions, the enzyme was observed in the columnar cells, especially in areas above the nucleus and in the gut lumen near the brush border (Lima

et al., 2003). Digestive α-amylase of *P. americana* was purified to homogenety and characterised by Due et al. (2008). Wang et al. (2000) inhibited amylase activity of the digestive tube of *P. americana* using synthetic polypeptides based on α-amylase inhibitor from the seeds of the Mexican plant *Hypochondriacus amarantus*. The results showed that the engineered chimera polypeptide huwentoxin-I (HWTX-I) exerted obvious inhibitory activity to cockroach α -amylase at pH 5.5 in the concentration of $9.5x10^{-5}$M. Endogenous carbohydrase activities localised in the foregut and midgut of the omnivorous *P. americana* were compared with the xylophagous *Panesthia cribrata*. The potential to hydrolyze cellulose and hemicellulose was higher in *P. cribrata*, while hydrolysis of starch and sucrose was more efficient in *P. americana* (Scrivener et al., 1998).

The digestive tract of the cockroach, *Blaberus craniifer* contains amylase, α- and β-glucosidases, β-fructofuranosidase, α- and β-galactosidases, but no cellulase. The most active site of hydrolytic action is the midgut, followed by the foregut, hindgut and salivary gland tissue (Banks, 1963).

Hindgut bacteria contribute significantly to the cellulose digestion in cockroaches (Bignell, 1977; Cruden and Markovetz, 1979). Although cellulase (endo-β-1,4-glucanase and β-glucosidase) activities appear to form part of the digestive complement of cockroaches (Wharton and Wharton, 1965), truly xylophagous or wood feeding species are in the minority. Cellulose digestion in the Australian wood-feeding cockroach *Panesthia cribrata* (Blaberidae) was the subject of considerable studies (Scrivener et al., 1998; Scrivener and Slaytor, 1994). Symbiotic gut microorganisms make no contribution to cellulose digestion in *P. cribrata*, mainly performed by an endogenous cellulase secreted by the midgut epithelium and salivary glands.

e. Digestion of proteins and digestive peptidases

Polyphagy, which is usually associated with the presence of complex digestive enzymes, is considered as an acute problem in insect pest control (Bown et al., 1997; Brito et al., 2001; Lemos and Terra, 1992; Jongsma et al., 1996). The interaction of insects with food sources has been a predominant factor in insect evolution (Christeller et al., 1989; Lemos and Terra, 1992; Terra and Ferreira, 1994). The wide range of the gut peptidases allow them to survive on a variety of foodstuffs (Shaw et al., 1965; Tsai et al., 1986). It is well documented that insects synthesize novel and/or different set of proteinases depending upon the food (Bown et al., 1997; Brito et al., 2001; Jongsma et

al., 1996; Patankar et al., 2001). Thus, detailed studies of digestive enzymes, including peptidases are crucial for understanding insect adaptations to their food sources and possibly also for developing insect control methods based on the digestion regulation. Several reports showed presence of multiple peptidases in insect gut and demonstrated that polyphagous species have a wider peptidase spectrum than the specialized ones (Bown et al., 1997; Jongsma et al., 1996; Brito et al., 2001). The digestive peptidases of insects catalyze the release of free amino acids from dietary proteins and thereby provide a supply of essential nutrients.

In brief, some characteristics of digestive peptidases utilized by insects are:

1. serine peptidases (e.g., trypsin-,chymotrypsin- and elastase-like) are generally active in the pH 7.0 - 10.0 range and are inhibited by such well-known inhibitors as Bowman-Birk, Kunitz, and lima bean inhibitor;
2. cysteine peptidases (e.g. cathepsin B and L) are generally most active in the mildly acid range in between pH 5.0 to pH 7.0. They are inhibited by heavy metals, cystatin, and E-64 inhibitor; and are enhanced by reducing agents;
3. aspartic peptidases (e.g., cathepsin D) are active in the acid pH range, generally below pH 4.5, and are inhibited by pepstatin A (Barrett, 1999; North, 1982).

Many peptidases are synthesized as inactive precursors or zymogens (Khan and James, 1998). Earlier attempts to show the occurrence of inactive precursors (zymogens) of insect digestive proteinases were unsuccessful (Applebaum, 1985; Graf et al., 1986). Barillas-Mury (1991) sequenced what seemed to be the precursor of midgut trypsin in *Aedes aegypti.* The sequence found was similar to that of most trypsins, although it was significantly different from the vertebrate trypsin precursors. Similar results were found with a putative trypsinogen from *Drosophila melanogaster* (Davis et al., 1985) and from *Simulium vitatum* (Diptera) (Ramos et al., 1993). These differences suggest that the processing of precursors of insect trypsins may be different from that in vertebrates. In accordance with these findings, there is evidence in *Tineola bisselliella* (Ward, 1975) and *Bombyx mori* (Eguchi, 1982) that soluble trypsin derives from membrane-bound forms. In *Erinnyis ello* (Santos and Terra, 1984, 1986) and in *Musca domestica* (Espinoza-Fuentes et al., 1987; Terra et al., 1988; Lemos and Terra 1992a, b) trypsin is synthesized in midgut cells in an active form, but is associated with the membranes of small vesicles. These vesicles then migrate to the cell apex and trypsin precursors are processed to a soluble form before being secreted. It seems that insects may control the

activity of their digestive peptidases even in the absence of inactive forms, by peptidase binding to the membranes until they are released into the lumen of the midgut.

Few reports are available on cockroach peptidases. Trypsin-, chymotrypsin-, and cysteine-like peptidases were detected in the midgut of a few cockroach species (Baumann, 1990; Elpidina et al., 2001a, b). Recently, Lopes and Terra (2003) have purified and characterized a single trypsin from the midgut of adult *P. americana* and revealed its biochemical properties. In another study, a clone from *B. germanica* cDNA library encoded a 24-amino acid signal peptide and a 328-amino acid mature protein (allergen Bla g2), which showed similarity to mosquito lysosomal aspartic peptidase, pepsin, cathepsin D and E, renin, and chymosin (Arruda et al., 1995). However, no homologous mRNA was detected in the American cockroach *P. americana*. The importance of this allergen in digestion has been further studied and a strong correlation between peptidase activity and the allergenicity of cockroach extracts demonstrated (Iraneta et al., 1999). Eleven proteinase activity bands were detected in the gut of American cockroach (Hivrale et al., 2005).

V. ENZYME INHIBITORS IN THE PEST CONTROL

Peptidase inhibitors (PIs) are considered as a part of alternate strategy to control the herbivorous insects via inhibition of their digestive enzymes (Haq et al., 2004). They are also effective against nematodes, viral, bacterial, and fungal pathogens..

The mode of PIs action on insects is based on the anti-digestive effect through proteolysis inhibition (Jongsma and Bolter, 1997) and also from the total increase of the organism fitness costs initiated by peptidase hyperproduction to compensate for the decrease of digestion efficiency. Generally, PIs ingestion reduces growth or developmental rates with some impact on mortality mainly described in first instar larvae (Broadway and Duffey, 1986).

However, PIs have often a minimal success on insects as a component of artificial diet or expressed in the transgenic plants (Carlini and Grossi de Sa, 2002). The lack of the effectiveness is based on the adaptive capacity of insect gut proteolytic system (Christou et al., 2006). Essentially, two types of resistance or adaptation to peptidase inhibitors have been described. The first one depends on the presence of peptidases which are insensitive to the action of the inhibitors. They may be constitutively expressed or induced in response to the inhibition of other enzymes to compensate for the loss of activity (Bayes et al., 2005, 2006; Bolter and Jongsma, 1995; Jongsma et al., 1995;

Lopes et al., 2004). The second mechanism depends on the production of peptidases which can degrade inhibitor (Girard et al., 1998; Giri et al., 1998; Ishimoto and Chrispeels, 1996; Michaud et al., 1995 a,b; Zhu-Salzman et al., 2003). Our success in using PIs to control herbivorous insects may be improved by:

1. isolating and designing small PIs domains that may function against a broad array of enzymes within a single subclass;
2. identification of digestive enzymes secretion regulating factors in the midgut (Broadway; 2000);
3. identification of a second generation peptidase inhibitors (novel PIs) from novel sources (unrelated organisms, insects or synthetic libraries) with maximum effectiveness against the selected target pests.

For example, elastase inhibitor isolated from haemolymph of *Manduca sexta* in preliminary bioassays showed potent insecticidal activity against the larvae of the same species (Kanost et al., 1989). The elastase inhibitor was also modified through site-directed mutagenesis to acquire activity against chymotrypsin or trypsin (Jiang et al., 1995). Both these inhibitors were used for transformation of tobacco or cotton. Plants modified by these inhibitors were more stable to attack of thrips *Frankliniella* spp and sweet potato whitefly, *Bemisia tabaci* by reducing pest reproduction by as much as 98% (Thomas et al, 1995). The use of novel inhibitors, such as the barley trypsin inhibitor (BTICMe) (Alfonso-Rubi at al., 2003), equistatin from the sea anemone (Gruden et al., 1998), other cystatins (Martinez et al., 2005) or synthetic constructs containing multiple inhibitors (Outchkourov et al., 2004) or inhibitors and lectins (Zhu-Salzman et al., 2003) might also prove useful.

The other new potential biopesticides - the bifunctional inhibitors like α-amylase/trypsin inhibitors from barley is of particular practical interest since transgenic plants expressing a molecule which inhibited both amylases and proteases of pests would be highly protected.

Another approach may be to express two or more PIs as a fusion protein. Expression of a cystatin and a serine PI in this way has been found to be successful against certain nematode pathogens (Urwin et al., 1998). Oppert et al., (2005) examined the effects of class specific inhibitors on gut proteolytic activity in *T. castaneum* larvae fed either E-64 (cysteine peptidase inhibitor) and/or STI (serine peptidase inhibitor). STI added to the diet had minimal effect on either the developmental time or proteolytic activity of *T. castaneum* larvae. However, after feeding on E-64 the dramatic shift from primarily

cysteine-type midgut digestion to serine was found. Only the using of both inhibitors prevented this adaptive shift and revealed substantial retardation of growth.

Sometimes the combined use of peptidase inhibitors with other insecticidal agents like *Bacillus thuringiensis* (*Bt*) Cry endotoxins shows the synergistic effect. Studies on the insecticidal activity of transgenic tobacco plants expressing both *Bt* insecticidal protein and cowpea trypin inhibitor (CpTI) genes against cotton bollworm (*Helicoverpa armigera*) revealed that it was more effective compared to transgenic tobacco expressing only *Bt* insecticidal protein gene. Besides the enhancement of insecticidal efficacy, insect adaptation to such transgenic *Bt* crops was also delayed (Fan et al., 1999).

These data suggest that PIs have the potential to be effective insecticidal proteins if insect adaptation to them can be overcome. By this reason studies of the organization of insect gut digestive proteolytic complex become very important for the prediction of possible adaptations and development of resistant crops for sustainable agriculture.

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CHAPTER 2

Proteinase, amylase, and proteinase-inhibitor activities in the gut of six cockroaches species

Proteinase, amylase, and proteinase-inhibitor activities in the gut of six cockroach species. Vinokurov K., Taranushenko Y., Krishnan N., Sehnal F. 2007. Journal of Insect Physiology 53: 794-802.

Representative species, two from each of the cockroach families Blattidae, Blattellidae, and Blaberidae, have similar morphology of the digestive tract but seem to differ in the physiology of digestion. The pH of crop and along the midgut varies in different species from 5.9 to 9.0 and the redox parameter from 10.1 to 12.9. Activities of proteinases and amylases in comparable gut regions differ among the species up to hundred times. Blaberidae exhibit distinct compartmentalization of the proteolytic and amylolytic activities; the former inreases, and the latter decreases from the crop to posterior midgut. Blaberidae are also distinguished by high potential of the salivary glands, crop, and midgut to inhibit subtilisin, trypsin, and chymotrypsin. Inhibitory activites in the species of other families are either absent or several orders of magnitude lower. The data indicate that organization of the digestive process diversified in Blaberidae from the two other families.

CHAPTER 3

Proteinase inhibitors from the cockroaches: perspective of their use in the plant protection (in Czech)

Inhibitory proteináz izolované ze švábů: perspektivy využití v ochraně rostlin

V005 – ZVYŠOVÁNÍ ODOLNOSTI PLODIN VŮČI ŠKŮDCŮM

INHIBITORY PROTEINÁZ IZOLOVANÉ ZE ŠVÁBŮ: PERSPEKTIVY VYUŽITÍ V OCHRANĚ ROSTLIN

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Úvod

Podstata trávení potravy u živočichů spočívá v jejím mechanickém zpracování, enzymatické hydrolýze organických molekul a v aktivitě mikroorganismů přítomných v trávicí soustavě. V hmyzím trávicím traktu se někdy nacházejí také inhibitory hydrolytických enzymů (Gooding 1974; Engelmann a Geraerts 1980; Stiles aj. 1991; Zhuzhikov 1997; Elpidina aj. 2001). Jejich úloha není zcela jasná. Je možné, že omezují působení trávicích enzymů, které by mohly poškodit střevní stěnu nebo symbionty. Nelze však vyloučit, že jsou ochranou proti patogenům, které používají specifické hydrolázy k překonání obranného systému hmyzího organismu. Ten se brání inaktivací takových hydroláz (Eguchi 1982; Yamashita a Eguchi 1987). Obdobný obranný mechanismus je často používán rostlinami, které vytvářejí inhibitory pro řadu enzymů. Produkce inhibitorů se zvyšuje při napadení rostliny hmyzem (Hilder a Boulter 1999) a zřejmě i vlivem patogenů. Eliminace této obrany činí rostliny zranitelnějšími vůči napadení herbivory (Royo aj. 1999). Mnoho rostlin, např. z čeledi Leguminaceae, více méně úspěšně používá tento obranný systém. S pomocí genetického inženýrství je možné vnést geny specifických inhibitorů proteáz z takových rostlin do genomů zemědělských plodin (Horsch aj. 1984), očekávaný biologický efekt však zůstává za očekáváním. Jedním z důvodů je schopnost hmyzu používat široké spektrum trávicích enzymů, které se mění podle složení potravy, a také schopnost potlačit produkci inhibitorů v rostlině (Girard aj. 1998; Zhu-Salzman aj. 2003; Bown aj. 2004). Rezistence hmyzu k inhibitorům enzymů produkovaných rostlinami není překvapují vzhledem ke ko-evoluci systémů rostlina-hmyz po několik set milionů let. Podobně je tomu v případě systémů rostlina-patogen.

Předpokládáme, že herbivorní hmyz a fytopatogeny, které mají schopnost překonat přirozený obranný systém rostlin, budou hůře vzdorovat látkám, se kterými se během své evoluce nesetkaly buď vůbec a nebo jen ve velmi omezené míře. To může platit pro inhibitory enzymů z jiných organismů než jsou rostliny – např. z vhodných druhů hmyzu. Silný obranný systém proti patogenům lze očekávat u hmyzu, který se živí rozkládajícími se zbytky potravy bohaté na mikroorganismy. Do této skupiny patří také švábi, kteří v přírodě povětšině konzumují složky hrabanky, ale mnoho druhů se stalo synantropními a požírají téměř vše. Jejich schopnost trávit různorodou potravu je patrná z přítomnosti 11 různých proteáz ve střevě *Periplaneta americana* (Hivrale aj. 2005). Krom trávicích enzymů obsahují některé části zažívací soustavy švábů i inhibitory hydroláz. Druhy švábů se podstatně liší v aktivitě trávicích enzymů i v účinnosti a množství inhibitorů. Např. *Nauphoeta cinerea* z čeledi Blaberidae obsahuje téměř 20.000 krát vyšší aktivitu inhibitorů než *Blatta orientalis* z čeledi Blattidae (Vinokurov aj. 2007). V rámci výzkumného centra 1M06030 se pracuje na identifikaci a charakterizaci inhibitorů proteináz *N. cinerea*, izolaci příslušných genů a přípravě rekombinantních inhibitorů v *Pichia pastoris*. Na základě biologické účinnosti rekombinantů bude rozhodnuto o klonování do rostlin.

CHAPTER 4

Peptidase inhibitors from the salivary glands of the cockroach *Nauphoeta cinerea*

Peptidase inhibitors from the salivary glands of the cockroach *Nauphoeta cinerea*. Taranushenko Y., Vinokurov K.S., Kludkiewicz B., Kodrík D., Sehnal F. 2009. Insect Biochemistry and Molecular Biology (accepted).

Inhibitory activity against subtilisin, proteinase K, chymotrypsin and trypsin was detected in the salivary glands extract and in saliva of the cockroach *Nauphoeta cinerea* (Blattoptera: Blaberidae). Purification by affinity chromatography followed by reverse-phase HPLC yielded from the salivary glands extract five subtilisin-inhibiting peptides with approximate molecular weights ranging from 5 to 14 kDa. N-terminal sequences and subsequently full-length cDNAs of inhibitors designated NcPIa and NcPIb were obtained. The NcPIa cDNA contains 216 nucleotides and encodes a pre-peptide of 72 amino acid residues of which 19 make up the signal peptide. The cDNA of NcPIb consists of 240 nucleotides and yields a putative secretory peptide of 80 amino acid residues. Mature NcPIa (5906.6 Da, 53 residues) and NcPIb (6713.3 Da, 60 residues) are structurally similar (65.4% amino acid overlap) single-domain Kazal-type peptidase inhibitors. NcPIa with Arg in P1 position and typical Kazal motif VCGSDstiochiometrically (1:1) interacted with subtilisin and was slightly less active against proteinase K. NcPIb with Leu in P1 and modified Kazal motif ICGSD has similar activity on subtilisin and about 8 times lower on chymotrypsin.

CHAPTER 5

Expression of *NcPiB* in the *E. coli* and *P. pastoris* expression systems (unpublished results)

Expression of NcPIb in the *E. coli* and *P. pastoris* expression systems

In previous work we have described purification, characterization and cDNA identification of two proteinase inhibitors from the *Nauphoeta cinerea* salivary glands. The objective of this research is to investigate the suitability of the *P. pastoris* and *E.coli* expression system as a means of recombinant expression of NcPIb.

Introduction

The physiological function of proteinase inhibitors in the digestive system of cockroaches remains unclear. However, its inhibition of subtilisin and proteinase K has led us to postulate a role of NcPIa and NcPIb in essential antifungal and antibacterial protection in cockroaches gut. To test this possibility, we decided to produce recombinant variant of NcPIb. The recombinant Kazal-type serine peptidase inhibitor r NcPIb (60 amino acid residues) from the *Nauphoeta cinerea* salivary glands, was prepared in the expression vectors *Escherichia coli* and *Pichia pastoris*.

Recombinant protein expression in *Pichia pastoris*

Forward primers F1 beginning with a *Xho*I restriction site and reverse primer F2 with a restriction site *Xba*I and GAT GGT GAT GAT GAT GTT encoded terminal hexahistidine was used to generate rNcPib. Plasmid pPICZαB (Invitrogen) with inserted PCR product was introduced into *Escherichia coli* strain DH5α. The transformants were selected on low-salt LB agar plates containing 25 μg/ml of Zeocin and the inserts were sequenced on the ABI Prism Sequencer (Perkin Elmer model 310). The clones containing the desired DNA template were used for the transformation by electroporation (Trans Porator Plus BTX, Invitrogen) of *P. pastoris* strain SMD 1168. Transformed clones were selected on YPD agar plates containing 100 μg/ml of Zeocin. Primers 5`AOX1 and 3`AOX1 (Invitrogen), which correspond to the 5` and 3` ends of the alcohol oxidase promoter (AOX1), were used in the sequencing reaction for screening construct insertion.

Yeast clones with verified inserts were grown in 200ml BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, $4x10^{-5}$ % biotin, 1% glycerol, and 100mM potassium phosphate buffer, pH 6.0) at 30 °C at 200 rpm agitation. After about 24 h, when the culture reached optical density (OD) 2.0 at A_{600} nm, the cells were centrifuged (3000 x *g* for 5min) and resuspended in BMMY (BMGY without glycerol) that was used in one-fourth of the original medium volume. Transcription controlled by the AOX1 promoter was activated by addition of methanol to a 5% of final concentration. Subsequent incubation at 30 °C with vigorous shaking was carried on for 4 days. The culture medium was then cooled to 4 °C and cleared from the yeast cells by centrifugation at 3000 x *g* for 10 min (Kludkiewicz et al., 2005).

Recombinant fusion proteins that contained the hexahistidine tag were pre-purifed by affinity chromatography on Ni-NTA resin (Invitrogen). A 5ml medium aliquot was brought to 10ml binding buffer (20mM Na_2HPO_4, 500mM NaCl at pH 7.4) and gently mixed with 200 µl Ni–NTA resin at 4 °C for 2 h. The resin with attached His-tag was spun down at 700 x *g* for 10 min and washed five times with 2ml binding buffer before the bound protein was eluted with four 0.2ml portions of the elution buffer (200mM imidazole in the binding buffer). The protein content of collected fractions was measured with bicinchoninic acid reagent (Walker, 2002). Fractions containing protein were analysed by SDS–PAGE (Laemli, 1970) on 18 % separating gel with subsequent Coomassie Blue R-250 staining. The inhibitor activity of purified rNcPib was assayed by the inhibition of subtilisin hydrolysis of substrate SAAPLpNA as it was described previously (Vinokurov et al., 2007). Unfortunately, recombinant protein did not exhibited any inhibition activity.

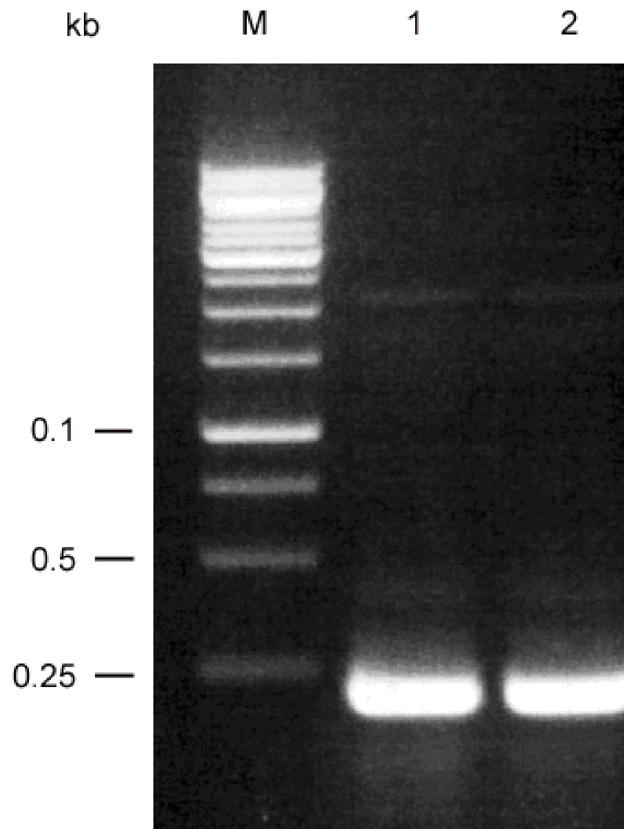

Fig. 1. Verification of construct insertion in the pPICZαB plasmid. Lanes 1 and 2, PCR amplification of the insert. Lane M, DNA markers (1 kb DNA Ladder; Fermentas).

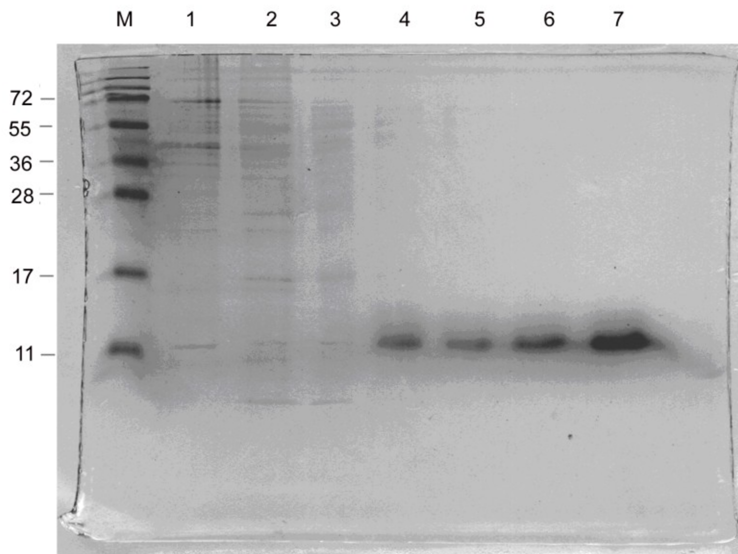

Fig.2. SDS-PAGE (18% gel) of rNcPIb in *P. pastoris.*

Tricine–SDS–PAGE: protein molecular mass standards (lane M); r NcPIb purified by affinity chromatography (lanes 1-4); whole culture medium of *P. pastoris* 72 hours after induction (lanes 5-7; 5,10,15 µl media, respectively).

Recombinant protein expression in *Escherichia coli*

The cDNA sequence coding for mature protein was amplified with a pair of primers, ProtB-Exp fw (5` – CAC CAT GAG AAC CTC TGT CGT CGT CC – 3`) and ProtB-Exp rev (5` – TGT TCA TTC TTC GTC GGT TTC CGC A – 3`) and subcloned into expression vector pET160/GW/D-TOPO (Invitrogen) (Fig. 3). The recombinant protein was expressed in *Escherichia coli* BL21 (Invitrogen) host cells grown at 37 °C in Luria-Bertani (LB) broth and protein production was induced with 0.8M IPTG (isopropyl-beta-D-thiogalactopyranoside; AppliChem). The bacterial cells were collected by centrifugation and the pellets were resuspended in three volumes of binding buffer (see above) and incubated with 0.2 mg/ml lysozym (Sigma) for 30min at room temperature. Subsequently, the suspension was sonicated on ice (150W, 6 x 10 sec) and centrifuged at 5,000 x g for 10 min. Recombinant fusion proteins that contained the hexahistidine tag on the C-terminal end were purified by Ni–NTA affinity chromatography (Invitrogen) following the manufacturer's instructions. Proteins were eluted with five portions of binding buffer containing 200mM imidazole. The aliquots were TCA-precipitated and analyzed by Coomassie Blue-stained SDS-PAGE (not shown). Expressed rNcPIb was detected in lysate and elution fraction by Western blot, using commercial antibody against the histidine tale (Fig. 4). Our preliminary experiments were carried out to find the optimal conditions for efficient expression, therefore, we compared profile of *E.coli* growth and made a induction time screening of rNcPIb expression in culture after three, four, five and six hours induction with IPTG. We found that maximal inhibitor activity reading in the culture was reached after 4 hours of the induction. Aliquots of lysate and every eluted fraction were also used for inhibitory kinetic experiments (Vinokurov et al., 2007) (Fig. 5).

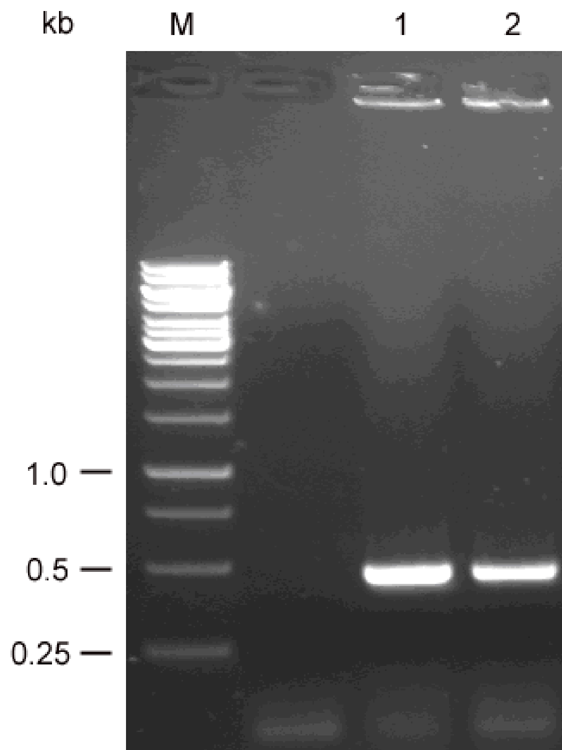

Fig. 3. Analyzing positive transformants in pET160/GW/D-TOPO plasmid. For PCR primers were used combination of the T7 Promoter sequencing primer and primer B rev (5`- GCTCTCACCAACCTCATAGG-3`). Lane M, DNA markers (1 kb DNA Ladder; Fermentas). Lanes 1 and 2, PCR amplification of the insert.

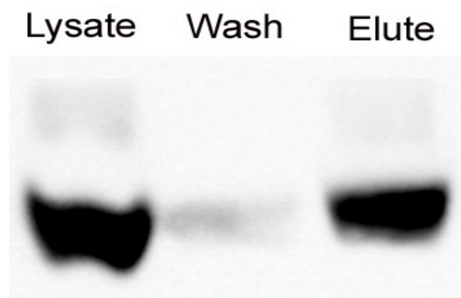

Fig. 4. Western blotting analysis. Proteins were separated by SDS-PAGE, and the gel were transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% milk in TBST and probed with mouse Anti - polyHistidine primary antibody (Sigma) (1:2000) and Anti-Mouse IgG Peroxidase (1:10 000) as secondary conjugates. rNcPib protein was detected by chemoluminescence using Amersham ECL Direct Nucleic Acid Labelling and Detection System.

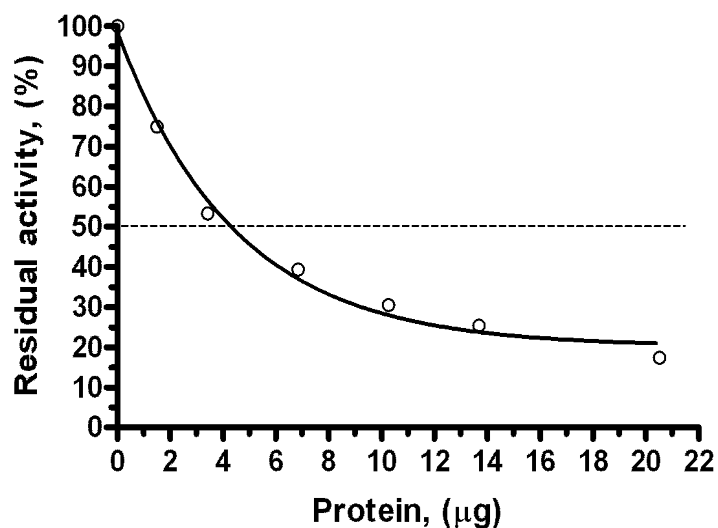

Fig. 5. Inhibitor activity detection against subtilisin in lysate of *E. coli* BL1 cells expressed recombinant protein NcPIb.

Conclusion

The classical Kazal-type peptidase inhibitors contain three disulphide bonds that are essential for proper folding and correct activity. However, the disulfide bonds in native eukaryotic proteins were often not accurately formed in prokaryotic organisms like bacteria. On the other hand, the presence of sugars decreased inhibition activity of several peptidase inhibitors (Dabich et al, 1993; Phadke et al., 1998; Kludkiewicz et al., 2005). Consequently, another advantage of *Pichia* centres on the type of glycosylation that results, generally yielding protein-bound oligosaccharides that are of much shorter chain length than found in *Saccharomyces cerevisiae*.

At first, we have selected the methylotropic yeast *Pichia pastoris* to express inhibitor B (NcPIb) since several reports have shown that this yeast are capable to effective and high-level expressing of proteinase inhibitors (Chen et al., 2004; Kludkiewicz et al., 2005). Despite our expectation the preparation of recombinant NcPIb (rNcPIb) with poly-his on C-terminus in *Pichia pastoris* has not been successful due to the unclear reasons that need further investigation.

Therefore, in our second access, we amployed rapid *E. coli* expressing system. This approach afforded us satisfactory levels of chemical active inhibitor.

However, in the near future, we would like to establish improvements in both expression systems and purify structure and biochemical active high yield of inhibitors.

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CHAPTER 6

Conclusions

- **Cockroaches from families with similar feeding habits have distinctly different organization of gut digestion**

Representative species, two from each of the cockroach families Blattidae, Blattellidae, and Blaberidae, have similar morphology of the digestive tract but seem to differ in the physiology of digestion. The pH of crop and along the midgut varies in species of the Blaberidae family (*Nauphoeta cinerea* and *Blaptica dubia*) from slightly-acidic (5.9-6.7) in crop and anterior midgut (AM) to alkaline (8.2-9.0) in the posterior midgut (PM). However, in the representatives of Blattidae (*Blatta orientalis* and *Periplaneta americana*) and Blattellidae (*Blattella germanica* and *Supella longipalpa*), pH is close to neutral along the entire gut. Activities of peptidases and amylases in comparable gut regions differ among the species up to hundred times. Blaberidae exhibit distinct compartmentalization of the proteolytic and amylolytic activities; the former inreases, and the latter decreases from the crop to posterior midgut. Blaberidae are also distinguished by high potential of the salivary glands, crop, and midgut to inhibit subtilisin, trypsin, and chymotrypsin. Inhibitory activites in the species of other families are either absent or several orders of magnitude lower. The data indicate that organization of the digestive process diversified in Blaberidae from the two other families.

- **Salivary glands of cockroaches *N. cinerea* (Blattoptera: Blaberidae) produce inhibitors active against bacterial peptidases**

Inhibitory activity against subtilisin, proteinase K, chymotrypsin and trypsin was detected in the salivary glands extract and in saliva of the cockroaches. The highest inhibitor activity was against microbial peptidases – subtilisin and proteinase K. The spectrum of subtilisin inhibitors revealed by reverse zymography included at least four electrophoretic fractions with Mw 14, 10, 7 and approx. 5 kDa in the salivary gland extract. The same electrophoretic pattern of inhibitors was found in the secreted saliva. Comparative analysis with crop, AM, and PM showed that these parts of the digestive tract contained only one fraction that inhibited subtilisin. This inhibitor has the same electrophoretic mobility as the main 14 kDa inhibitor fraction found in the salivary glands and saliva.

- **Two steps chromatographic purification yielded from the salivary glands extract five pure peptidase inhibitors, two of which were found to belong to the Kazal family**

Purification of the salivary glands extract by affinity chromatography followed by reverse-phase HPLC yielded five subtilisin-inhibiting peptides with approximate molecular weights ranging from 5 to 14 kDa. N-terminal sequences and subsequently full-length cDNAs of inhibitors designated NcPIa and NcPIb were obtained. The NcPIa cDNA contained 216 nucleotides and encoded a pre-peptide of 72 amino acid residues of which 19 made up the signal peptide. The cDNA of NcPIb consisted of 240 nucleotides and yielded a putative secretory peptide of 80 amino acid residues. Mature NcPIa (5906.6 Da, 53 residues) and NcPIb (6713.3 Da, 60 residues) were structurally similar (65.4% amino acid overlap) single-domain Kazal-type peptidase inhibitors.

Purified inhibitors NcPIa and NcPIb were highly active only against microbial peptidases. The expression level of both inhibitors in the salivary glands was not influenced by starvation. However, the incubation of salivary glands in the presence of serotonin that is known to stimulate the secretory activity of the central cells of salivary acinus, significantly enhanced the contents of corresponding transcripts. This enhancement was correlated with a rise of inhibitor activity secreted from the serotonin-stimulated glands into the incubation medium.

WHAT REMAINS TO BE DONE – PLAN OF FUTURE WORK

- ## Why saliva contain a spectrum of peptidase inhibitors?

NcPIa and NcPIb are closely related structurally but differ in activities against proteinase K and chymotrypsin. I want to identify the cDNAs and to characterize activities of the three remaining peptidase inhibitors from the saliva of *N. cinerea.* Methods described in this paper will be used. Structural and functional comparison of all inhibitors is expected to reveal if they evolved by gene duplication and why and to what extent they have diversified.

- ## Possible function(s) of saliva inhibitors

Based on the preferential activity of NcPIa and NcPIb against microbial peptidases from the clan SB (subtilisin, proteinase K), we propose that these inhibitors may have a protective role. Cockroaches usually feed on substrates that are highly contaminated with microbial and fungal microflora. Proliferation of microbes in the digestive tract and especially in the crop where food can be stored up to 100 hours is probably under control of several factors, including well known lysozyme and the newly identified peptidase inhibitors .It is possible that the inhibitors from saliva represent major defense against microbial degradation of ingested food and/or against uncontrolled proliferation of potentically harmful microorganisms. I plan to prepare recombinant inhibitors in *Escherichia coli* and examine their activities on selected bacteria and fungi.

- ## Practical potential of identified peptidase inhibitors

Specific activities of recombinant inhibitors will be compared with the activities of commercial inhibitors. If the activities of new inhibitors will be comparable or higher, attempt will be made to design and produce modifications that may be even more active. These would be offered for industrial use or for plant transgenesis that would provide protection against some plant pathogens.