Control of the release of digestive enzymes in the cricket *Gryllus bimaculatus* and the fall armyworm, *Spodoptera frugiperda*

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To my wife
Hingfene Gong-Non Moundou
and children
Reggabe Digali and Lwatchebe Digali

Introduction

Insects represent the dominant group in the world fauna (80% of known animal species in the world are insects) and most are beneficial to the ecology and the economy. Most flowering plants depend on insects for their pollination (the values of insect-pollinated crops was of the order of US $17, 000 million for a total of about 90 crops in the USA in the year 1986). Virtually all predators and parasites of pest insects are other insects. However, insects are severe pests in agriculture, destroying 15% of total production, representing over US $ 100 billion, and are vectors of major sickness such as malaria, dengue, sleeping disease, and yellow fever.

During the last century a huge amount of chlorinated hydrocarbon and organophosphate pesticides were used to control the populations of insect pests. These pesticides are potent nerve toxins, functioning by inhibiting the action of acetylcholinesterase in nerve cells. Such effects are the results of changes in the kinetics of the Na^+ and K^+ ion fluxes across the nerve cell membranes and subsequent interference with nerve synaptic transmission. This toxic mechanism is nonspecific, affecting both insects (target species) as well as all nontarget species. These pesticides are contact poisons in all animals. They are fat soluble and are absorbed through the body wall when the insect walks on the treated foliage, or when it moves through the thatch and soil. Contact poisons can also enter through the digestive tract when the insect feeds on treated plant tissues.

In the last 30 years synthetic or natural compounds, which can interrupt or inhibit the life cycle of the target insects, were developed. These chemicals are known as insect growth regulators (IGR), which depend on using the insects own hormones or hormone analogs to control pest populations. Insect growth regulators disrupt the normal development and reproduction of insects by mimicking juvenile hormone (JH) and/ or moulting hormone (20-hydroxyecdysone). This means they have very low toxicity to non-arthropod animals, however they are seldom used today, because these products are quickly degraded in the environment and are quickly metabolised and/or excreted in the insect.

Transgenic plants provide an attractive alternative to the use of chemical pesticides and herbicides and could contribute to the production of crop varieties that are inherently resistant to their major insect pests. Therefore, in recent years, intense research has been focused on gene products (proteins) with insecticidal activities. *Bacillus thuringiensis* (BT) produce crystal proteins (cry), which punctures the insects

midgut wall and cause uncontrolled leakage and death in all lepidopteran larvae tested (Pigott et al., 2008). When the cry genes are incorporated into plant genome, the cry endotoxins are very effective and very specific.

The first generation of pesticides (organophosphate, chlorinated hydrocarbons) posed a great hazard because they inhibit synaptic transmission in essentially all organisms and accumulate in adipose tissues. They are toxic to non target animals and totally disrupted the environment. The second generation of pesticides (insect growth regulators) are not toxic to vertebrates, but IGR effectiveness was limited. The third generation of pesticides are essentially peptides that for the most part disrupt some aspect of digestion. One example is the cry proteins (above), a second example are enzyme inhibitors. Plants produce a broad range of ihnhibitors of insect digestive enzymes such as soybean trypsin inhibitor, bestatin and wheat α-amylase inhibitor. In general it is not practical to apply cry proteins, neuropeptides or enzyme inhibitors to plants as a control method, because of high costs, quick degradation, and very low uptake by the insect. Therefore, the genes for these materials must be incorporated into the plant genome.

There are a number of plant protease inhibitors (see review, Fan and Wu, 2005) which bind to endo- and exopeptidases in insects and effectively inhibit growth, and likewise a number of plant α-amylase inhibitors (see review, Franco et al., 2002). In recent years it has been shown that members of several families of insect neuropeptides, including allatostatins (AS) and allatotropins (AT), control not only the juvenile hormone (JH) biosynthesis and release (regulating growth and reproduction) but also the release of digestive enzymes and gut myoactivity in many insects (Fusé et al., 1999; Hill and Orchard 2005). Insertion of genes into crop plants that encode protease inhibitors or neuropeptides would be worth the effort, but only if more detailed information on the action of these peptides indicates effective *in vivo* regulation of digestive functions. Therefore, a major goal of my research is to evaluate the effect of inhibitors and neuropeptides on the release of digestive enzymes in model insects.

The first goal was to elucidate the effect of specific nutrients [glucose, maltose, starch, bovine serum albumin (BSA), peptone and amino acids mixture] on the release of digestive enzymes in the two model insects *Spodoptera frugiperda* (Lepidoptera, Noctuidae) and *Gryllus bimaculutus* (Ensifera, Gryllidae). The second goal was to examine the endogenous control of digestive enzyme release via

neuropeptides (allatostatins and allatropin). The third goal was to examine plant based enzyme inhibitors and compare them to endogenous enzyme inhibitors. As model insects I used the cricket *G. bimaculatus* (an omnivore with hemimetabolic development) and the fall armyworm *S. frugiperda* (a herbivore with holometabolic development) because the cricket represents the basic primitive form of the insect digestive system, and the fall army worm represents a specialized herbivore of considerable economic importance. It was also important that the experimental animal was easy to rear on a defined diet, and that they are large enough to dissect.

Digestive systems in insects

The digestive systems of vertebrates and insects are very similar both in structure and function (Penzlin, 1999). Despite the diversity of feeding habits, three basic divisions occur in all insects: the foregut which serves mostly for storage, the midgut where enzymes are secreted and digestive products are absorbed, and the hindgut where undigested food and metabolic wastes are compacted, dehydrated, and egested (Nation, 2002; Dettner and Peters, 2003). The foregut and hindgut are ectodermal and the epithelium secretes a chitinous cuticle continuously with the exoskeleton. The midgut (ventriculus) secretes a delicate protective peritrophic envelope around the food, which also regulates flow of digestive enzymes (Dettner and Peters, 2003). The midgut wall is a single layered epithelium with an extensive microvilli on the luminal side, which increases surface area for maximum absorption of nutrients.

The foregut of *G. bimaculatus* consists of a pharynx, an oesophagus, a very large crop, and thick-walled muscular proventriculus (Woodring and Lorenz, 2007). The crop stores a large mass of freshly ingested food and enzymes originating in the midgut initiate digestion. Most enzyme release and nutrient absorption occurs in the large lateral caeca arising from the anterior end of the ventriculus. Typical of terrestrial insects, the rectum very efficiently resorbs water to produce a very dry fecal pellet. The flow of nutrients through the digestive tract of *G.bimaculatus* is regulated by the proventriculus, which effectively triturates the partially digested food coming from the crop and shoves the mushy nutrient mass into the ventriculus (Woodring and Lorenz, 2007). Nutrients are pressed into the caeca, and the remaining indigestible food is shoved into a tube formed by the peritrophic membrane

(PM). A countercurrent flow of enzymes has recently been described in crickets (Biagio et al., 2009)

The major deviations from the primitive digestive system occur in fluid feeders, that is, with phytophagous or with blood sucking insects. The larvae of the fall armyworm, *S. frugiperda*, represent a typical phytophagous insect, which have a simple straight-through type of gut without outpockets (caeca). Such a digestive tract is well designed for transport of large masses of food with low nutrient quality. The incompletely digested food mass of moist plant material passes rapidly through the gut and water is not resorbed in the rectum (Nation, 2002). Ingested food is not stored, so the crop is greatly reduced. The ventriculus comprises about 80% of the total gut and the surface area is increased by a complex series of ring like folds (Eaton, 1988). A well developed peritrophic membrane is formed in the anterior end of the ventriculus (Harper and Granados, 1999), and it creates an endo- and exoperitrophic space, in which a separation and recycling of digestive enzymes occurs (Terra et al., 1979; Terra and Ferreira, 1994; Ferreira et al., 1994b; Terra et al., 1996b; Bolognesi et al., 2001).

In *S. frugiperda* trypsin and α-amylase are secreted into the ectoperitrophic space and diffuse into the endoperitrophic space, whereas aminopeptidase and disaccharidases are secreted into the ectoperitrophic space and remain attached to the membrane complex of the gut epithelium. Aminopeptidase is both bound to the microvillar membranes and present as a soluble enzyme trapped in the cell glycocalyx of the ventriculus (Klinkowstrom et al., 1994). Partially digested food moves inside the PM (endoperitrophic space) posterior, while outside the PM (exoperitrophic space) fluid containing partially or completely digested food plus digestive enzymes move anterior. The enzymes involved in initial digestion are secreted by the epithelium and pass through into the anterior endoperitrophic space where they bind to nutrients. The enzyme-substrate complexes are too large to diffuse back across PM, however, as the food is digested the molecular size of the food particles decreases until these along with the enzymes can then pass back into the exoperitrophic space. Along with resorbed water, the enzymes and oligomeric molecules flow anterior where terminal digestion (to monomers) and absorption occur (Terra et al., 1996 a, b). The enzymes diffuse back into the anterior endoperitrophic space and a new cycle starts (Bolognesi et al., 2001).

Digestive enzymes in insects

Enzymes are catalysts that regulate all reactions in all cells in the body (Lehninger, 1956; Penzlin, 2003). All enzymes are proteins, whose sequence and the spontaneous folding patterns and twists create specific 3-dimensional shapes. The particular folding pattern of each enzyme gives each enzyme a distinct characteristic and function. By disruption of the specific folding pattern, for example at temperatures above 45°C, enzymes lose their ability to function, becoming inactivated or are destroyed. The digestive enzymes are extracellular hydrolases, which cut a nutrient molecule at a specific site by inserting a water molecule.

G. bimaculatus and *S. frugiperda* contain the typical array of insect digestive enzymes in their digestive tracts. The chemical structure and catalytic action of these enzymes are quite similar in all animals (Penzlin, 2003). However, the storage of digestive enzymes, the activation of enzymes, the site where the enzymes are released varies considerably between vertebrates and insects (Nation, 2002). The hormonal and neural regulation of digestive enzyme release in vertebrates is well understood, but is in general not as well understood in insects (Penzlin, 1991). In the omnivorous cricket *G. bimaculatus,* the caecal region of the midgut is the primarily site for the secretion of digestive enzymes. Proteases and amylase flow anteriorly from the caeca into the mostly empty crop on day 1, and carbohydrate and protein digestion starts as soon as food is present (Woodring et al., 2007).

In the midgut of the phytophagous larvae of *S. frugiperda*, aminopeptidase and part of amylase, carboxypeptidase, dipeptidase, and trypsin are bound to the microvillar membranes (Ferreira et al., 1994b). Major amounts of soluble dipeptidase, and maltase are trapped in the cell glycocalyx; and only soluble carboxypeptidase, amylase, and trypsin occur in intracellular vesicles. Aminopeptidase are mostly bound to cells membranes in the ectoperitrophic space and a minor amount incorporated in the peritrophic membrane. Amylase and trypsin are found primarily in the endoperitrophic space after diffusing through the PM. The gradient is maintained by binding of amylase and trypsin to substrates in the endoperitrophic space.

Proteases are enzymes acting on peptide bonds either in the middle of the protein (endopeptidases) or at either end (exopeptidases). Endoproteases are divided into three classes. 1) The serine proteases are characterized by the presence of a serine and histidine at their active site. Examples are trypsin, chymotrypsin, elastin, and subtilin. 2) The cysteine proteases possess a cysteine at the active site

and are inhibited by mercurial compounds. Examples are cathepsin B and cathepsin L. 3) The aspartic proteases have a pH optimum below 5, due to the involvement of a carboxyl residue in catalysis, for example pepsin. Exopeptidases hydrolyse single amino acids from the N-terminus (aminopeptidase) or from the C-terminus (carboxypeptidase) of the peptide chain and those enzymes that hydrolyse specific dipeptides are called dipeptidases (see review Fan and Wu, 2005).

Typsin is a member of the serine proteinase superfamily, which have a common catalytic triad of the specific residues: serine, histidine and aspartic acid (Klein et al., 1996). Trypsin is highly specific for the positively charged side chains of leucine and arginine (Kraut, 1977). Trypsins occur in almost all insect species. The trypsins isolated from lepidopteran insects have a higher pH optimum, corresponding to the higher pH values found in their midguts (Ferreira et al., 1994a). Cleavage specificity of trypsins from several insects show that these enzymes are similar (but not identical) to that of vertebrates trypsins. For example, insect trypsins are not activated or stabilized by calcium ions (Lemos and Terra, 1992), in most cases are unstable in acid pH (Sakal et al., 1989) and have different sensitivities to natural trypsin inhibitors (Purcell et al., 1992). The molecular weight of insect trypsins lie between 21 and 28 kDa and they do not require activation once secreted (Terra et al., 1996a). Other serine proteases such a chymotrypsin, elastase, and subtilisins were not considered in this dissertation because of time limitations and the known similarity in the mode of action and release.

Aminopeptidases and exopeptidases are widely distributed as both extra- and intracellular enzymes in all animals, including insects (Hooper and Lendeckel, 2004). As integral membrane or cytoplasmic proteins they play important roles in various biological processes such as protein processing and turnover, and various pathological disorders regulation of peptide hormone action (Brownlees and Williams, 1993; Huang et al., 2009). The aminopeptidases are metalloenzymes, and they occur in all animals and are especially active in the digestive tract of most insects (Terra et al., 1996a; Woodring et al., 2007). Insect aminopeptidases have alkaline pH optima (range 7.2 - 9.0), irrespective of the pH of the midgut lumen in which they occur (Ferreira and Terra, 1985), and a molecular weight in the range of 90 - 190 kDa.

The cellular carboxypeptidases of vertebrates are very important in the biosynthesis of insulin and function in blood clotting, growth and wound healing. In insects they play a role in many physiological processes including the maturation of

neuroendocrine peptide precursors (Terra et al., 1996a). As digestive enzymes they hydrolyze, along with the aminopeptidases, the peptides resulting from endopeptidase action to liberate free amino acids to complete the digestion process and produce molecules, which can be absorbed by the gut (Bown and Gatehouse, 2004). Carboxypeptidases were not included in my dissertation research because they have a low activity in *Spodoptera* and *Gryllus* (unpublished results), and the literature indicates little difference in mode of action and release to that of aminopeptidases.

Insect **carbohydrases** are classified into two broad categories. The first group includes enzymes which cleave internal bonds in polysaccharides, and that are usually named from their substrates, for example by amylase, cellulase, pectinase and chitinase. The second category includes glucosidases (maltase, trehalase, fructosidase, xylosidase etc.) that catalyse the hydrolysis of terminal bonds in oligosaccharides and disaccharides (Franco et al., 2002). The resulting monosaccharides glucose, fructose, galactose etc. are directly absorbed in the midgut.

Insect α-amylases (α-1,4-glucan-4-glucanohydrolases) are a group of glycoside hydrolases that are widely distributed animal tissues, which catalyse the hydrolysis of the α-(1,4) glycosidic linkage found in starch, glycogen and other polysaccharides (Franco et al., 2002). These enzymes are well adapted to the slightly acidic to neutral conditions found in the more anterior regions of the midgut in many insects (Buonocore et al.,1976). The central calcium ion stabilises the 3-dimensional structure of α-amylase and protects it against digestion by proteases (Stein and Fischer, 1958).

Lipids consist of a large and heterogeneous group of substances that are relatively insoluble in water but readily soluble in apolar solvents. **Lipases** hydrolyse ester bonds in lipids containing fatty acids as fats, phospholipids, glycolipids and waxes before they are absorbed (Terra et al., 1996a). Insect lipases**,** triacylglycerol hydrolases, preferentially hydrolyse the outer ester links of triacylglycerols (TAG) and release free fatty acids, diglyceride, monoglyceride, and glycerol (Secundo et al., 2006). Insect cellular lipases in the fat body are needed for the hormone stimulated adipokinetic hormone (AKH) release of diacylglycerides into the hemolymph, which is the essential flight fuel for most insects (Wheeler and Goldsworthy, 1983). Insect lipases preferentially cleave fatty acids from the α-positions (Hoffman and Downer,

1979), prefer unsaturated fatty acids (Weintraub and Tietz, 1973) and are activated by calcium ions (Gilbert et al., 1965), thus resembling the action of mammalian pancreatic lipase.

Insect midgut triacylglycerol lipases have been studied in relatively few insects and only in crude preparations. Other than in vertebrates, fat micelles are not formed and bile salt emulsification does not occur (Penzlin, 2003). The phytophagous larval *Manduca sexta* appears to completely hydrolyze triglycerides in the midgut, and absorbs the released fatty acids into the hemolymph (Tsuchida and Wells, 1988). However, as in vertebrates the transport of lipids into the hemolymph in insects also involves reconstitution to triglycerides in the ventricular cells (Turunen and Chippendale, 1989).

Release of digestive enzymes

There is a vast literature regarding the stage and age variation of digestive enzymes in whole gut preparations of all groups of insects (see Terra, 1996 a, b, Chapman, 1998). There is, however, relatively little known about the factors controlling the release of digestive enzymes in insects. This knowledge is a prerequisite for developing methods of control of pests based on inactivation of digestive enzymes. The chief aim of my dissertation therefore was to determine what extrinsic factors (**nutrients**) and intrinsic factors (**neuropeptides**) are most important in the regulation of enzyme release. The second aim was to examine the action of exogenous enzyme inhibitors, occurring in the food, and compare them with the occurrence and action of endogenous inhibitors secreted by the gut.

Neuropeptides

A number of neuropeptides have been isolated in various insect orders, chiefly from the brain and other parts of the nervous system and from the digestive tract. These peptides are multifunctional and are best known for their effect on growth and reproduction, controlling primarily the release of juvenile hormone (JH) from the corpora allata (CA). They either stimulate (**allatotropin**, AT) or inhibit (**allatostatin**, AS) the secretion and release of JH (Hoffmann et al., 1999; Gäde, 2002). Neuroendocrine cells are distributed throughout the midgut, and in recent years it has been shown that the same neurohormones that regulate JH release also affect

digestive processes such as release of digestive enzymes and gut myoactivity (Nachman et al., 1997; Fusé et al., 1999; Penzlin, 1999).

Type-A allatostatins (Grybi-AS and Spofr-AS), also called cockroach allatostatins, are characterized by the common C-terminal pentapeptide sequence Y/FXFGL-amide (Stay et al., 1991). They were first isolated from the brains of the cockroach *Diploptera punctata* (Woodhead et al., 1989) and have subsequently been found in multiple forms in most insect orders (Nässel, 2002; Gäde, 2002). The cricket *G. bimaculatus* hormone precursor contains at least 14 putative allatostatins (Meyering-Vos et al., 2001). These AS have a strong allatostatic effect on the corpora allata *in vitro* in crickets, cockroaches, and termites, but not in other insects (Hoffmann et al., 1999). I used Grybi-AS 5 (DRLYSFGLamide), an allatostatin that not only has a strong allatostatic effect, but it and related peptides are also myoactive and stimulate release of amylase in cockroaches (Fusé et al., 1999). Immunocytochemical studies showed that type-A AS in cockroaches are widely distributed in the body, not only in the central nervous system but also in peripheral nerves and in midgut endocrine cells (Stay, 2000). In cockroaches, a number of AS inhibit spontaneous contraction of muscles of the hingut and in the antennal pulsatile organ (Lange et al., 1993). In *S. frugiperda,* the gene encoding a family of nine to ten peptides (type-A Spofr-AS) was isolated from brain cDNA (Abdel-latief et al., 2004). There is evidence of allatostatic activity for these peptides in the adult moth. I used two of the possible peptides deduced from the precursor molecule, Spofr-AS A5 (ARAYDFGLAamide) and Spofr-AS A6 (LPMYNFGLamide), to test their effect on digestive enzyme release and on gut myoactivity.

To date, only one allatotropin family (Manse-AT) (TARGF/Yamide) is known, and it functions in a number of Lepidoptera to stimulate the release of JH from corpora allata. First isolated from the head of the moth *Manduca sexta* (Kataoka et al., 1989), Manse-AT mRNAs were detected in the brain and suboesophageal ganglion as well in cells of the abdominal ganglia (Veenstra et al., 1994). This suggests multiple physiological functions beyond allatotropic effects, such as functions in regulation of digestion. Manse-AT (GFKNVEMMTARGFamide) has also been isolated from head extracts of *S. frugiperda* (Oeh et al., 2000). In larvae of *S. frugiperda*, three mRNA isoforms of the *Manse-AT* gene are expressed in brain, digestive tract, and reproductive organs (Abel-latief et al., 2004). To date there is no

information on the functions in the larvae or in regulation of digestive enzyme release.

Inhibitors

Considerable efforts are being made to explore the use of plant genes encoding insecticidal proteins in developing insect resistance in susceptible crop plants (Lawrence and Koundal, 2002; Ferry et al., 2004). To date several different classes of plant proteins have been shown to be insecticidal towards a range of economically important insect pests when tested in artificial diets or in transgenic plants (Gatehouse and Gatehouse, 1998). Plant protease and α-amylase inhibitors (PI) form the main classes of insecticidal proteins, and they function by forming complexes with digestive enzymes which are highly stable and dissociate very slowly (Gatehouse et al., 1999; Morton et al., 2000; Fan and Wu, 2005). In plants which produce them, they are thought to provide a form of natural defence against herbivorous insects (Broadway and Duffey, 1986). The possible role of PIs in plant protection was envisaged as early as 1947 when Mickel and Standish observed that the larvae of certain insects were unable to develop normally on soybean products (Haq et al., 2004). Soybean (Kunitz) trypsin inhibitor (SBTI) was the first PI isolated and characterized (Kunitz, 1948; Fan and Wu, 2005).

Most plants PIs vary from 8 to 20 kDa (Hung et al., 2003), and they have a high content of cysteine residues that form disulfide bridges and confer resistance to heat, extremes in pH, and proteolysis (Richardson, 1991). The gene sequence for SBTI is known and it has been inserted into a number of different plants including potato, tobacco and rice (Fan and Wu, 2005). The Kunitz type trypsin inhibitor (SBTI) is the trypsin inhibitor I used in my dissertation research. It has a low molecular weight (25 kDa), and is quite resistant to high termperatures (Kunitz, 1948). It is readily available in a purified crystalline form. The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^7 to 10^{14} M at neutral pH values), thus effectively blocking the active site (Terra et al., 1996a). Thus PIs directly mimic a normal substrate for the enzyme, but do not allow the normal enzyme mechanism of peptide bond cleavage to proceed to completion i.e., dissociation of the product (Walker et al., 1998).

Relatively few exopeptide inhibitors in plants have been described (Bayes et al., 2005). Bestatin is an inhibitor of exopeptidases extracted from *Streptomyces*

olivoreti and its structure was determined to be N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (Umezawa et al., 1976). The molecular weight is only 345 Da. Bestatin inhibits metalloproteases and is selective for aminopeptidases including leucine aminopeptidase and alanyl aminopeptidase use at 1-5 mM (Fan and Wu, 2005).

α-Amylase inhibitor, from *Triticum aestivum* (wheat) kernels which are particularly rich in inhibitors of α-amylases, affect carbohydrate digestion in many insects and mammals (Baker and Lum, 1989; Gomez et al., 1989). Some of these are selective for insect enzymes, in that they inhibit α-amylases from insects strongly but inhibit mammalian salivary or pancreatic α-amylases only weakly or not at all (Feng et al., 1995). Plant based α-amylase inhibitors have been separated into three major families of inhibitors (Petrucci et al., 1974). The family with the smallest molecular weight (12 kDa) are monomeric proteins, the inhibitors of molecular weight of 24 kDa are dimeric proteins. The third family of inhibitors, which have a molecular weight of 60 kDa, are not well defined (Buonocore et al., 1980). The dimeric α-amylase inhibitor I used in my dissertation is one of the most studied inhibitor from the cereal- α-amylase inhibitors family (Franco et al., 2002).

Research gaps and the aims of this thesis

There is a vast literature regarding the stage and age variation and the effect of feeding on the digestive enzyme activity present within the gut lumen representing all groups of insects (Applebaum, 1985; Chapman, 1998; Terra et al., 1996a, 1996b). There is, however, relatively little known about the factors controlling release of digestive enzymes in insects (Lehane et al., 1996; Blakemore et al., 1995; Lwalaba et al., 2009). This knowledge is a prerequisite for developing methods of control of pests based on inactivation of digestive enzymes.

The chief aim of my dissertation was to determine what extrinsic factors (nutrients) and what intrinsic factors (neuropeptides) are most important in the regulation of enzyme release. There are relatively few studies addressing the direct effect of food (grains, plants, meat, etc.) in the gut on the release of digestive enzymes (prandial regulation), and very few studies on the direct effect of specific nutrients (protein, amino acids, starch, maltose, fat, etc.) on enzyme release. Aside from the effect of proteins on protease release in cockroaches (Engelmann and

Geraerts, 1980) and *Stomoxys calcitrans* (stable fly) (Blakemore et al., 1995) there is no knowledge of the effect of carbohydrates or lipids on the release of proteases, amylases or lipases. Essential for such investigations is a reliable system for incubation of midgut tissues.

The action of insect neuropepeptides on development and reproduction, water balance and digestion was summerized in several reviews (Gäde et al., 1997; Nässel, 2002; Gäde and Hoffmann, 2005). The effect of several neurohormones on the release of amylase and maltase was described for a cockroach (Fusé et al., 1999) and for a locust (Hill and Orchard, 2005). But the effect of neuropetides on the release of other enzymes in crickets or in any phytophagous insect remains unknown.

Meyering-Vos et al. (2001) identified a gene encoding 14 putative allatostatins (AS) in *G. bimaculatus* and five of these were previously isolated by Lorenz et al. (1995) by HPLC. The Grybi-AS precursor is expressed most strongly in the brain, subesophageal ganglion and in the midgut caecum (Meyering-Vos and Hoffmann 2003). In mated females the AS type-A may regulate JH biosynthesis during vitellogenesis, egg maturation and oviposition, respectively. However its effect on digesting enzymes in *G. bimaculatus* was not investigated. Likewise, using molecular biological techniques, Abdel-latief et al. (2004) found the gene sequences of two allatostatins (Spofr-AS5, AS6) and an allatotropin (Manse-AT) in *S. frugiperda*. Manse-AT strongly stimulates, and all tested Spofr-AS inhibit the biosynthesis of JH in adult *S. frugiperda* (Oeh et al., 2000). Therefore, we know which neurohormones occur in our model insects, *G. bimaculatus* and *S. frugiperda*, however, the effect of these neuropeptides on gut myoactvity or on the release of the digestive enzymes was not known. Therefore, this was an important aim of my research.

A further aim of the dissertation was to investigate the action of exogenous enzyme inhibitors (those inhibitors that occur in the food), and compare them with the occurrence and action of endogenous inhibitors that are secreted by the gut. Because of the great importance of protease inhibitors (PI) for agriculture, there is a vast literature on the effect of protease inhibitors from plants (see review Fan and Wu, 2005) on protease activity in the gut. Likewise, a great amount of information on the successful adaptation of insects to PIs has accumulated (Jongsma et al., 1995; Broadway, 1995; Bown et al., 1997; Gatehouse et al., 1997; Brito et al., 2001; Brioschi et al., 2007). However, there are almost no studies on the effect of PI or

other inhibitors or mixtures thereof on the release of digestive enzymes. Such studies can actually be best carried out with short-term (3 days) feeding in the last larval stage and only with tissue incubation, and this is what I did.

Except for the works of Engelmann and Geraerts (1980) and Vinokurov et al. (2007) there is very little known about endogenous inhibitors in insects, and essentially nothing is known about the factors regulating their release. The functions of these endogenous inhibitors is completely unknown, but my investigation of the regulation of the release has suggested possible new function in insects.

In order to answer the questions regarding the regulation of the release of digestive enzymes in insects, as elucidated above, the following experiments were performed:

1. Determination of the release *in vitro* of amylase, trypsin, aminopeptidase, and lipase from flat-sheet caecal preparations in response to feeding of a control diet, a non-nutrient diet and starvation in young adult females of *G. bimaculatus* and in L6 larvae of *S. frugiperda*.
2. Determination of the effect of various nutrients in the incubation medium (specifically proteins, peptides, amino acids, starch, maltose and glucose) on the release of amylase, trypsin and aminopeptidase from isolated flat-sheet preparations of the caecum (*G. bimaculatus*) and the ventriculus (*S. frugiperda*).
3. Determination of the effect of cricket allatostatin Grybi-AS5 on the release of digestive enzymes, and determine the relative importance of prandial and hormonal control in the cricket *G. bimaculatus.*
4. Determination of the effect of armyworm allatostatin (Spofr-AS5 and Spofr-AS6) and tobacco hornworm allatotropin (Manse-AT) on the release of enzymes from incubated midguts (flat-sheet preparations) of *S. frugiperda.*
5. Determination of the dose-dependent inhibition resulting from acute feeding exogenous inhibitors (soybean trypsin inhibitor, aminopeptidase inhibitor bestatin, and wheat amylase inhibitor) in last instar larvae (L6) of *S. frugiperda*. Possible cross inhibition between inhibitors in *S. frugiperda* was also investigated.
6. Determination of the presense of endogenous inhibitors in L6 *S. frugiperda* larvae in comparison to the exogenous inhibitors. Suggest a role for the occurrence of endogeneous enzyme inhibitors in insects.

Synopsis

The basic aim of my dissertation research was first, to determine relative importance of feeding various diet, specific nutrients in the diet (extrinsic), and neuropeptides (intrinsic) on the release of enzymes from the midgut tissue of the cricket *Gryllus bimaculatus* and of the fall armyworm, *Spodoptera frugiperda,* and second, to compare the effects of exogenous protease and amylase inhibitors on the production, release, and lumen contents of trypsin, aminopeptidase and amylase in the larvae of the pest species (*S. frugiperda*).

In the **first paper** (Physiological Entomology 34, 144-151, 2009) I found that in *Gryllus bimaculatus* the release of digestives enzymes into the caecum is regulated by feeding, by specific nutrients and by the neuropeptide allatostatin 5 (Grybi-AS5). The amount of all three enzymes released into the caecum of fed animals was consistently 20-70% more than in unfed animals but a considerable amount of trypsin and amylase was released even after 24 h without food. In unfed animals, feeding always stimulated within an hour an increase in enzyme release over the basal (unfed) levels, which continued over the next 4 h. The released amylase, trypsin and aminopeptidase activity into the incubation medium after only 30 min was double the enzyme activity within the caecal cells. The lipase release on the other hand was less than a quarter of the activity measured in cell homogenates. This confirms our earlier results (Woodring et al., 2007) that in crickets there is a basal level of enzyme activity in the gut in unfed animals, and that feeding leads to a general increase in enzymes.

Not only was the enzyme release higher in fed animals compared with unfed animals, but also the composition of the food had a profound effect on the ratio of the digestive enzymes released. Feeding stimulated the release of digestive enzymes in response to a specific ratio of nutrients, which led to a different ratio of digestive enzyme release. A high nutrient component (for example protein) in the food did not result necessarily in a correspondingly high digestive enzyme for that component (trypsin). An increase over basal secretion rates of amylase, trypsin, and lipase occurred in crickets fed cat food (high protein), but not when fed rabbit food (high carbohydrate). In order to explain this anomaly, specific nutrients were added to the incubation medium of isolated flat-sheet preparations of the digestive caeca.

A flat-sheet preparation is made by cutting a section of the gut lengthwise so that both sides are exposed to the incubation medium. It could be demonstrated that such preparations are stable, secrete enzymes over a period of several hours. The

caecum is composed of a single layer of several types of epithelial cells. Enzymes are secreted from the luminar side of the zymogen cells and neurohormal receptors are present on the hemolymph side. Nutrient receptors are located on the luminar side of neuroendocrine cells and neurohormones are secreted from the hemolymph side. The flat-sheet caecal preparation that I developed for this study allowed me to demonstrate the paracrine mechanism of enzyme release in response to nutrients and the endogenous control of enzyme release via neurohormones (allatostatins, allatotropin).

There was no significant stimulation of amylase release in response to low concentrations of starch to the incubation medium, but there was a strong dose-dependent increase in amylase release, up to a 175% increase at maltose concentrations of 8.0 mg maltose/ml incubation medium, in both fed and starved crickets. Glucose also strongly stimulated amylase release. Trypsin or aminopeptidase release was not affected by starch or sugars in the incubation medium. There are no polysaccharide (starch) receptors known in any animal, but the sugar receptors present in the epithelium of *G. bimaculatus* seem to be similar to those in vertebates. In the case of the cricket, sugar binding leads to a strong stimulation of amylase release. The slight increase in amylase activity at higher starch concentration probably was not due to starch, but rather the result of traces of glucose in the starch, which initiated digestion, and the resulting additional glucose or maltose stimulated amylase release.

There was a slight increase in trypsin release in response to 0.4 to 0.6 mg/ml peptone, which is a partial digest of casein containing a mixture of short length polypeptides and amino acids, in the incubation medium, but at very high concentrations of peptone (>6 mg/ml) there was an inhibition of trypsin release. Similarly, with lower concentrations of bovine serum albumin (BSA) there was no effect on trypsin release, but at concentrations above 2 mg/ml a strong inhibition of trypsin release was observed. This unexpected inhibition of trypsin at high BSA concentrations is probably due to the protein (BSA) blocking or altering access to the enzyme active site, as observed with plant protein inhibitors (Fan and Wu, 2005). Perhaps a kind of “excessive substrate inhibition” occurs in *G. bimaculatus*, where excess BSA irreversibly binds to the trypsin active site. Excess substrate inhibition occurs in approximately 20% of all enzymes. There was basically no stimulation of exopeptidase (amino- and carboxypeptidase) release in response to BSA, peptone or

to a mixture of amino acids. The observed inhibition of trypsin release at higher peptone concentrations is also probably due to excess substrate inhibition.

Similar to the study on the control of the release of digestive enzymes in the cricket *G. bimacualtus* (first paper), the main purpose of the **second paper** (Archives of Insect Biochemistry and Physiology, in press, doi:10.1002/arch.20332) was to describe the regulatory role of various nutrients and neuropeptides on the release of enzymes from the ventricular tissue (midgut) in the larvae of the fall armyworm, *Spodoptera frugiperda*. A second aim was to determine the effect of these same neuropeptides on the myoactivity of the digestive tract. The results demonstrate that larval age**,** food consumed, and the source of the enzymes (lumen or cells), all influence the release of digestive enzymes into the lumen. It must be emphasized that most published data on digestive enzymes deal with the standing enzyme activity found in the lumen, and very little is known about the release.

The amount of enzyme released (activity) from the ventricular cells during the 3 days of the last larvae stadium (L6) increased from day 1 to the maximal activity on day 3, and then dramatically decreased on day 4 (prepupae) as the digestive tract emptied and started to disintegrate. The total activities of all four enzymes studied were almost 15-times higher in the ventriculus than in the ileum, which suggests that an efficient counter-current flow of enzymes occurs in the ventriculus of S*. frugiperda*, as is described by Bolognesi et al. (2001), and this reduces the loss of important digestive enzymes via egestion. A conclusion drawn from the observation that the enzyme activity in the ventriculus of nutrient-fed larvae was much higher than those fed the non-nutritive cellulose diet is that it is the nutrient composition and not merely the bulk that stimulates enzyme release over that of the basal levels.

Aminopeptidase activity was much higher in the cell fraction and amylase and trypsin much higher in the lumen. Typical of lepidopteran larvae (Ferreira et al., 1994b; Lenz et al., 1991; Ortego et al., 1996), trypsin and amylase occur primarily in the endoperitrophic space and the aminopeptidase is bound to the ventricular cell membranes.

The total lipase activity of S*. frugiperda* is low, but the lumen fraction of the total is relatively high (Lwalaba et al., 2009). This is the first report of high lipase activitiy in the gut lumen of Lepidoptera larvae. Based on studies with radiolabelled trioleine, dietary triacylglycerol in *Manduca sexta* larvae is completely hydrolyzed to

free fatty acids in the lumen before absorption into the cells (Tsuchida and Wells, 1988). We suggest, that because lipase and amylase have similar molecular weights (50-60 kD) it would be expected that soluble lipase also passes into the endoperitrophic space at the anterior end of the ventriculus. Amylase is secreted at the anterior end of the ventriculus and passes through the pores of the peritrophic membrane into the endoperitrophic space (Ferreira et al., 1994 a,b).

In continuously feeding insects, such as the caterpillar *S. frugiperda*, there are few benefits to be gained from regulated secretion of digestive enzymes. A basal release rate of enzymes into the ventriculus independent of the presence of food in the gut is found in many insects (Chapman, 1985), however, feeding almost always leads to an increase in the release and amount of enzymes in the gut (Applebaum, 1985; Woodring et al., 2007). In the cockroach *Leucophaea maderae*, feeding stimulated the production of proteases in the midgut (Engelmann and Geraerts, 1980). The amylase release is three times higher in fed than in unfed larvae of *S. frugiperda*, but trypsin release (quite unlike the cockroach) is the same for both fed and unfed larvae. Both enzymes are immediately released after synthesis into the lumen (Ferreira et al., 1994 a,b), which means that the synthesis rate must be related to nutrients in the gut. But why does amylase release vary with the feeding and trypsin release does not? A rationale based on food utilization can be hypothesized. The primary nutrient in the diet of the caterpillar is carbohydrate, much of which after digestion is absorbed, then converted to lipids and stored in the fat body. The adult moth subsequently uses this lipid as a flight fuel (Wheeler, 1989). Reduced dietary carbohydrate simply means less lipid reserve, and it would it be wasteful to produce large amounts of amylase when food is in short supply. Therefore, the larvae can afford to adjust the amylase release to the carbohydrate intake. Proteins, on the other hand, are essential for larval growth, and in the adult moth egg production depends entirely on proteins stored in larval fat body (Sorge et al., 2000). The caterpillar, therefore, can not afford to allow any dietary protein to pass undigested through the gut. Therefore, it is advantageous to maintain a constant level of trypsin release in the event that even a small amount of protein is ingested. Regulated secretion of digestive enzymes in insects, in which enzymes are synthesized and stored and then released only in response to massive amounts of food, occurs in some intermittent feeders (such as mosquitoes) (Lehane et al., 1996).

Whether the enzyme release rate is sufficient to maintain the standing amount present in the lumen was determined in the following manner. The time of food passage through the ventriculus, based on feeding L6 larvae carmine red colored diet (n=4), averaged 3 h (180 min). The standing amylase activity in the ventricular lumen was 340 µg maltose/min, and the secreted activity of the incubated flat-sheet prepared ventriculus was 12 µg maltose/min. Therefore, in 3 h (= 180 min) 72 µg maltose/min was secreted, which was about 20% of the standing amylase activity in the lumen. The standing trypsin activity in the ventriculus was 3.8 units/min, and the secreted activity was 0.11 units/min. In 3 h 0.66 units/min was secreted, which was about 17% of the standing trypsin activity. The standing lipase activity was 3.7 nmol/min. In 3 h 7.8 nmol/min lipase activity was secreted, which was more than the standing lipase activity. The standing aminopeptidase in the lumen was only 1.8 µmol/min. The very small aminopeptidase activity found in the incubate was probably released from damaged cells and not from secretion, because almost all aminopeptidase is membrane-bound. There was 10-times more amylase and trypsin in the lumen of the ventriculus than in the ileum, thus, the relatively high secretion rate of enzymes along with relatively little loss via egestion indicates that the secretion of enzymes is sufficient to maintain the standing concentrations in the lumen.

One can not determine the role of food or nutrients on enzyme release or synthesis by simply measuring the enzyme activity in the lumen, but rather a direct measure of enzyme secretion (release) by the ventricular tissues is required. In order to precisely determine the role of feeding on the increased secretion of enzymes over that of basal levels, specific nutrients were added to incubated flat-sheet preparations of the ventriculus. A concentration of 0.5 mg/ml or higher of maltose or glucose in the incubation medium resulted in an up to 350% elevation of amylase secretion. The maximum effect of starch on amylase release was a modest 30-40% and only at higher concentrations (>0.5 mg/ml). The larvae of the fall armyworm are plant-feeders, and simple sugars and polysaccharide intake is therefore high. In the current study, glucose and maltose provide a strong signal to release amylase, which is the normal way to stimulate amylase release in animals. There is no evidence in vertebrates or insects of starch or other polysaccharide receptors on gut neuroendocrine cells that induce amylase release. The presence of starch in the incubation medium, however, appears to only mildly stimulate amylase release in S.

frugiperda, and this is most likely due to a minimum digestion to maltose or a small amount of sugar in the starch tested, and it is the maltose or glucose that stimulates amylase release and not the starch. Similar to other insects, sugars bind to receptors in the apical membrane of neuroendocrine cells in the epithelial layer of the ventriculus, and induce the release of neuropeptides at the basal end into the hemolymph. A binding of sugars to the apical (lumen) end of the gut neuroendocrine cells is postulated in G. *bimaculatus*, which induces the release of neuropeptides from the basal end into the hemolymph (Woodring et al., 2007). Specific neuroendocrine cells in the gut epithelium of cockroaches and locusts release several kinds of neuropeptides that stimulate the release of amylase (Fusé et al., 1999; Sakai et al., 2004; Hill and Orchard, 2005).

The protein BSA had no effect on trypsin, and peptone (a mixture of small polypeptides) caused only a moderate increase ($p<0.05$) in trypsin. The trypsin release is not elevated by feeding in S. *frugiperda*. Therefore, it is not surprising that bovine serum albumen (BSA) has no effect on release, and that peptone or a mixture of all 20 amino acids has only a weak effect in the incubated flat-sheet preparations. BSA in the bloodsucking fly *(Stomoxys*), on the other hand, has a strong stimulating effect on the release of trypsin (Blakemore et al., 1995), which makes sense in an animal with sudden and massive input of protein.

The effect of nutrients on the release of lipases was not studied because of the very low activities. The aminopeptidase activity in the incubation did not result from secretion, but rather from broken cells. Almost all aminopeptidase remains bound to the external cell membrane (lumen side) and very little is released into the lumen (Ferreira et al., 1994 a,b).

The gene sequence for Manse-AT and Spofr AS A5 were recently identified in *S. frugiperda* (Abdel-latief et al., 2004), therefore the effect of these specific neuropeptides on the release of amylase, trypsin and aminopeptidase was investigated. The allatostatin Spofr-AS A5 inhibited the *in vitro* release of amylase and trypsin by the ventriculus, and the allatotropin Manse-AT significantly stimulated amylase and trypsin release at low concentrations. Neither the AS nor the AT had any effect on aminopetidases. The neurohormones AT and AS are small peptides that bind to receptors on the hemolypmh side of the zymogen cells (enzyme-producing cells) and induce either an inhibition or stimulation of the release on the

lumen side. One of the first examples was the stimulation of amylase release in response to AS, as was shown in the cockroach *D*. *punctata* (Fusé et al., 1999).

Interestingly, in almost all other Lepidoptera larvae an *in vitro* contraction of the foregut is seen (Duve et al., 1999; Matthews et al., 2007). However, in *S. littoralis* (Matthews et al., 2008) and in S. *frugiperda*, foregut motility was not observed. Therefore, in the current study the ileum was employed to assay myoactivity. Increased contraction of ileal muscles was observed only in a resting ileum with no spontaneous contractions, that is zero beats/min. Concentrations of Manse-AT greater than 10^{-6} M elevated the contractions from 0 beats/min to over 20 beats/min. Interestingly, the recovery back to 0 beats/min was graduate and required several transfers to fresh HGR (high glucose ringer) over 3-4 min, indicating that the peptide must be rinsed from the receptor site.

Inhibition of the contraction rhythm was observed only in an ileum that had started to beat spontaneously. The rate ranged from 15 to 30 beats/min, but once started the rate was constant for any single ileum over a 5 to 20 min period. Therefore, the amount of inhibition as a percent change from the control (initial rate) in this time span was consistent and repeatable. The ileum was very sensitive to inhibition by Spofr-AS A5 at all concentration over 10^{-8} M, showing an immediate and complete inhibition (0 beats/min). Recovery in fresh HGR required only 20-30 seconds, indicating a weaker receptor binding than that with AT (above). A WX_6W allatostatin (AS type-B) inhibits gut myocontraction in another lepidopteran species, *M*. *sexta* (Blackburn et al., 1995). In conclusion, Sporf-AT stimulates digestive enzyme release and myoactivity in *S. frugiperda*, and Spofr-AS A5 inhibits both enzyme release and myoactivity.

In the **third paper** (Journal of Insect Physiology, in review) the effects of exogenous and endogenous protease and amylase inhibitors on the production and secretion of digestive enzymes in the larvae of *S. frugiperda* was investigated. It has been long known that digestive enzymes are inhibited by a wide variety of inhibitors occurring in plants (Franco et al., 2002; Fan and Wu, 2005). A strong inhibition of trypsin activity by the general serine protease inhibitor SBTI, aminopeptidase by bestatin, and amylase by the wheat α-amylase inhibitor was observed in the lumen contents of L6 larvae of *S. frugiperda*. An age-dependent effect was apparent (with constant inhibitor concentration), whereby three days of feeding of inhibitor reduced

enzyme activity in the lumen (higher inhibition) more than one day of feeding. A distinct dose-dependent inhibition was also apparent after three days of feeding, which was also reported in other lepidopteran larvae (Paulillo et al., 2000; Brioschi et al., 2007).

With regards to inhibition of trypsin in tissue homogenates, both soybean trypsin inhibitor (SBTI) and N-α-tosyl-L-lysine chloromethyl ketone (TLCK) were effective. Bestatin and wheat amylase inhibitor had no effect on the secretion or tissue activity of aminopeptidase or amylase, respectively. SBTI strongly inhibited trypsin release in the tissue extracts, especially at high concentration (1200 µg/larva/day). At concentrations of TLCK >400 µg/larva/day a feeding inhibition was observed, and as a result (less TLCK consumed) no further trypsin inhibition was seen.

Whereas acute feeding (3 days) of inhibitors had only moderate effects on typsin activity in tissue extracts or no effect on aminopeptidase and amylase activity, these same inhibitors when added directly to the cellular extracts (*in vitro*) strongly inhibited all enzymes down to only 5-24% of control values (Table 3). Thus it was clear that the inhibitors studied inhibited specifically the enzymes (endogenous) release by the tissues and not just the exogenous enzymes used for the enzyme assays.

With regards inhibition of the release of enzymes, only TLCK was effective. This is the first direct evidence of inhibition of enzyme release in insects. Neither aminopeptidase nor amylase release is affected by the corresponding inhibitors employed. Inhibition of enzyme secretion by an ingested inhibitor implies either a direct effect (entry into the cell) or indirect effect (docking on receptors) on the cell production (synthesis) or release. Evidence for the effect of inhibitors on cellular enzyme production and release is seen in the increased trypsin activity in the gut of *S. frugiperda* and *Heliothis virescens* in response to chronic feeding of SBTI (Brito et al., 2001; Brioschi et al., 2007).

A cross reaction of one inhibitor on a different enzyme has been reported. In *Teleogryllus commodus*, where SBTI increased aminopeptidase activity (Burgess et al., 1994), as did a barley trypsin inhibitor in S. *exigua* (Lara et al., 2000). In our study SBTI fed to L6 larval *S. frugiperda* induced a moderate elevation ($P<0.05$) of aminopeptidase in the lumen, and the more potent protease inhibitor TLCK strongly elevated ($P<0.01$) aminopeptidase activity in both the lumen and in the tissue

extracts. Thus, aminopeptidase showed a cross reaction *in vivo* in response to both protease inhibitors (SBTI and TLCK). Bestatin, an aminopeptidase inhibitor, however, showed no cross reaction to *in vivo* trypsin inhibition. The cross reaction of SBTI and TLCK on aminopeptidase activity indicated an altered synthesis of one enzyme in response to a product reduction of a different enzyme. A molecular basis of crossover effects can be seen in *Helicoverpa armigera*, where specific inhibitors increased chymotrypsin mRNA but trypsin mRNA levels decreased (Gatehouse et al., 1997), or in *Trichoplusia ni*, where a procarboxypeptidase is activated by trypsin (Wang et al., 2004).

The difficulty in demonstrating the presence of endogenous inhibitors, specifically those secreted by the gut itself, is the high concentrations of endogenous enzymes present in the gut lumen. I solved this problem by irreversible inactivation by means of heat treatment. It was assumed that the endogenous inhibitors are as heat resistant as the demonstrated resistance of the exogenous inhibitors, which were used for the enzyme assays. The first demonstration of endogenous gut protease inhibitors was described in tissue extracts and lumen contents from the midgut of *Leucophaea maderae* (Engelmann and Geraerts, 1980). They also used boiled extracts to inactivate endogenous enzymes, and found up to 80% protease inhibition in the posterior half of the midgut. Endogenous inhibitors in whole midgut extracts (epithelium plus lumen contents) are also reported by Elpidina et al. (2001) and Vinokurov et al. (2007) in the cockroach *Nauphoeta cinerea*.

Heated tissue extracts in diet fed L6 *S. frugiperda* larvae showed less trypsin activity (78%), and much less aminopeptidase activity (47%) compared to controls (100%), which demonstrates the presence of endogenous inhibitors in the ventriculus. The unheated tissue extracts showed more trypsin (143%) and much more aminopeptidase (332%), a result of the endogenous enzyme activities. Since the tissues are thoroughly rinsed, exogenous inhibitors associated with the food are removed, and only endogenous inhibitors are to be found in the heated tissue extract and no active enzymes. In the unheated tissue extract the endogenous trypsin was still active and when combined with the exogenous bovine trypsin an increase instead of a decrease in total enzyme activity was seen. The same reasoning applies to aminopeptidase and amylase inhibitors present in the tissues extracts.

Endogenous enzyme activity is much higher in the lumen than in the tissues. Heated lumen contents showed much less trypsin (65%), and aminopeptidase (79%)

activity, clearly an indication of endogenous inhibitors. The unheated lumen contents showed very high trypsin activity (426%) because both endogenous and exogenous trypsin was present. There was basically no difference in the endogenous trypsin inhibitor activity in diet fed, cellulose fed or unfed L6 larvae. It should be noted that there could not be any exogenous inhibitors in the lumen of cellulose fed or unfed larvae.

Lumen contents run on an analytical SDS-PAGE gel showed the presence of an endogenous inhibitor, and the same lumen contents run on a gelatine containing SDS-PAGE gel demonstrated the binding of an endogenous protease inhibitor to the gelatine substrate.

The situation with endogenous aminopeptidase inhibition is different because most aminopeptidase is not released into the lumen, as are trypsin and amylase, but rather it remains bound to the cell membranes (Ferreira et al., 1994 a,b). The membrane bound aminopeptidases of heated tissue extracts were inactivated, and the endogenous aminopeptidase inhibitors were greatly reduced ($P<0.01$) in tissue extracts. The PM is part of the lumen contents, and it contains some aminopeptidase (Ferreira et al., 1994 a), and a moderate amount ($P<0.05$) of endogenous aminopeptidase inhibition occurs in heated lumen contents. In diet fed larvae the endogenous inhibitor strongly reduced ($P<0.01$) aminopeptidase in the tissue, less so in cellulose fed larvae, and not at all in starved larvae. In cellulose-fed and unfed larvae the aminopeptidase inhibition was undetectable in the lumen.

Endogenous amylase activity was studied only in fed larvae. Heated tissue extracts and lumen contents of L6 larvae showed less ($P<0.05$) amylase activity (85%) compared to controls, clearly indicating an endogenous amylase inhibition. The unheated tissue extracts and lumen contents showed more amylase (142%), indicating the high levels of endogenous amylase in the lumen.

It was demonstrated that trypsin digests both human salivary amylase as well as the amylase present in the lumen of *S. frugoperda* larvae. About 10% of the amylase is digested over a period of 30 min, which in fed larvae is quickly replaced. However, starved larvae only secrete about 20% of the controls and trypsin digestion of amylase could present problems.

Several functions of the endogenous protease inhibitors in the digestive tract of cockroaches have been suggested. Elpidina et al. (2001) suggest the endogenous protease inhibitors might protect amylases in the anterior midgut of *N. cinerea*,

however, this seems unnecessary because divalent ions confer a general resistance of α-amylases to proteolytic degradation (Stein and Fischer, 1958). Other authors suggest that endogenous protease inhibitors might be important as protection against fungi and bacteria that employ subtilisins to attack insects (Vinokurov et al., 2007; Taranushenko et al., 2009). Another possible function of endogenous protease inhibitors in insects might involve the protection of the epithelium when dietary protein is scarce. Trypsin is continuoulsy released in fed and unfed S. *frugiperda* (Lwalaba et al., 2009). In vertebrates an inactive trypsinogen is released in response to cholecystokinin (CCK), but a trypsinogen has never been found in insects. My hypothesis is that endogenous inhibitors, especially trypsin inhibitors, are essential to protect the delicate ventricular epithelium again the digestive action of trypsin (which is always present) when food is scarce or lacking.

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Control of the release of digestive enzymes in the caeca of the cricket *Gryllus bimaculatus*

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Abstract. In *Gryllus bimaculatus*, more digestive enzymes (amylase, trypsin, aminopeptidase) are secreted in the caecum of fed crickets than in unfed crickets, but the enzymes are released continuously at a basal rate in unfed animals. The rate of synthesis of the enzymes appears to parallel their rate of release. Digestive enzymes are released in response to a specific ratio of nutrients, although a high nutrient component in the food does not necessarily induce a high digestive enzyme release for that component. Rinsed flat-sheet preparations of the caecum are incubated with specific nutrients (carbohydrates and proteins) and various concentrations of a neuropeptide (type-A allatostatin), which affects generally the basal rates of secretion. Both maltose and glucose increase the release of amylase *in vitro*, but starch produces an inhibition of amylase release at lower concentrations. Bovine serum albumin (BSA), peptone and a mixture of amino acids have almost no effect on the release of aminopeptidase or carboxypeptidase, and only low concentrations of peptone increase trypsin release. High concentrations of both BSA and peptone strongly inhibit trypsin activity, perhaps by excess substrate binding to the trypsin active site. The allatostatin Grybi-AST 5 elevates the release of amylase *in vitro*, but not of trypsin or aminopeptidase, in 2-day-old fed females. In the caeca from 1-day-old unfed crickets, both amylase and the trypsin release are stimulated in the presence of AST 5. The paracrine AST 5 is probably released from the gut endocrine cells and binds to the enzyme-producing caecal cells.

Key words. allatostatin, aminopeptidase, amylase, diets, lipase, nutrients, trypsin.

Introduction

In vertebrates, the release of digestive enzymes is regulated by feeding, acting through a feedback to the brain that controls digestion directly via the visceral nervous system and indirectly via paracrine hormone release (Penzlin, 1996). Based on ultrastructural studies of midgut cells, a continuous release of digestive enzymes is indicated in most insects, and a regulated release of digestive enzymes (with stored enzymes in the cytoplasm) is assumed to occur essentially only in discontinuous feeders, such as blood sucking insects (Lehane *et al.*, 1996). Both the amount and the ratio of nutrients in the gut play an important role in the release of digestive enzymes, whereby more enzymes are generally released in fed insects than in unfed insects (Chapman, 1985; Woodring *et al.*, 2007). A direct neural regulation of digestive enzyme release in insects is considered to be unlikely (Lehane *et al.*, 1996; Sehnal & Zitnan, 1996; Chapman, 1998) and, therefore, the only possible mechanisms controlling the release are hormonal, paracrine or prandial (Lehane *et al.*, 1995).

To date, there is no available evidence for the hormonal control of digestive enzyme release in insects. An inhibition of trypsin biosynthesis (not release) via blocked transcription is known for the 'trypsin modulating oostatic factor' in mosquitoes, biting flies and fleas (Borovsky & Mahmood, 1995; Borovsky, 2003). A paracrine mechanism requires endocrine cells in the gut, which occur in all the insects investigated, and are usually concentrated in the midgut epithelium (Montuenge *et al.*, 1989; Sehnal & Zitnan, 1996). Many neuropeptides (FMRFamides, allatostatins, sulfakinins and others) are identified through antibody reactions in the enteric nervous system and in the endocrine cells of the gut (Sehnal & Zitnan, 1996). These affect both gut musculature via the enteric nervous

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system (Lange *et al.*, 1995) and digestive enzyme release in a paracrine way (Fusé *et al.*, 1999; Harshini *et al.*, 2002b; Hill & Orchard, 2005; Sakai *et al.*, 2006). A prandial mechanism (also termed a secretogogue mechanism) refers to the direct binding of a nutrient element to a digestive enzyme-producing cell causing the release of an enzyme; however, there is no convincing evidence of such a mechanism in insects (Lehane *et al.*, 1995).

There are many examples of ingested nutrients, particularly proteins in blood feeding insects, that stimulate the release of trypsin (Lehane *et al.*, 1996), and these most likely involve a paracrine mechanism. Endocrine gut cells are distinguished from other gut cells by the presence of peptidergic secretory granules of which there are two types in insects. In the open type, the apical end of the cell reaches the gut lumen and, in the closed type, a luminary contact is lacking (Sehnal & Zitnan, 1996). Gut endocrine cells usually have basal cytoplasmic processes from which the (neuro)peptides are released, and typically more than one peptide is produced and released. In *Gryllus*, large numbers of long open type secretory cells occur at the junction of the caeca and ventriculus (Sehnal & Zitnan, 1996).

A gene encoding 14 putative allatostatins (AST) is expressed most strongly in the brain of *G. bimaculatus* (Meyering-Vos *et al.*, 2001) and the isolation by HPLC of five of these is reported in this species (Lorenz *et al.*, 1995). The AST precursor is expressed most strongly in the brain, subesophageal ganglion and in the caecum. In all other tissues (including the other parts of the gut), the precursor is expressed at much lower levels (Meyering-Vos & Hoffmann, 2003).

In the present study, the release *in vitro* of amylase, trypsin and aminopeptidase from flat-sheet caecal preparations is investigated in response to feeding various diets and to the presence of various nutrients in the incubation medium, as well as the effect of allatostatin Grybi-AST 5. Possible mechanisms of digestive enzyme release (i.e. hormonal, prandial or paracrine) are discussed in light of the effect of nutrients and the neuropeptide AST 5 on enzyme release.

Materials and methods

Experimental animals

The Mediterranean field cricket *G. bimaculatus* de Geer (Ensifera, Gryllidae) was raised under an LD 16 : 8 h photoperiod at 27 °C as described previously (Lorenz *et al.*, 1997). Newly-emerged females were isolated within 1 h of the imaginal moult (long before they started to feed) and were designated 0-day-old crickets. Control 2-day-old unfed females always had access to water but no food. Fed 2-day-old females received a mixed diet consisting of ground rabbit, rat and cat food (4 : 2 : 1, w/w; Altromin, Germany) with a nutrient value of 40% carbohydrate, 25% protein and 6% lipids. When food is available, most females started feeding within 5–6 h of the final moult, and the crop is already half full 1 h later (Woodring & Lorenz, 2007). The sunflower seed diet was prepared by grinding seeds with a coffee bean grinder.

Tissue preparation and incubation

The cricket midgut consists of a pair of very large, internally folded caeca at the anterior end and a relatively short and narrow ventriculus (Woodring & Lorenz, 2007). Animals were pinned dorsal side up onto a piece of styrofoam and cut open from the periproct to the pronotum. The caecum was removed, cut open and rinsed three times with low glucose Ringer (10 mg glucose 100 mL^{-1} of *Gryllus* Ringer). The Ringer consisted of 8 g NaCl, 0.4g KCl and 0.4 g $CaCl_2$ per liter brought to a pH of 7.2 with 1 g Hepes. Contraction of the muscles of the opened caecum led to the formation of an open, cup shaped structure with the lumen side outermost and the hemolymph side inner most, which was designated a flat-sheet preparation (Blakemore *et al.*, 1995). Both sides of the caecal epithelium were equally exposed to the medium during incubation.

The effect of nutrients on the *in vitro* release of enzymes from the caecum was tested by addition of appropriate dilutions of stock solutions of starch (5 mg mL^{-1}), maltose (10 mg mL^{-1}), glucose (10 mg mL^{-1}), bovine serum albumin (BSA) 10 mg mL^{-1}, peptone (casein) (10 mg mL^{-1}) or a mixture of 20 amino acids (10 mg mL^{-1}) dissolved in 300 μL of the incubation medium (low glucose Ringer) for 30 min at 30 °C without shaking or aeration. The effect of neuropeptides on the release *in vitro* of enzymes from the caecum was tested by addition of appropriate dilutions of 10^{-3} M stock solution of Grybi-AST 5 (DRLYSFGLa) in 300 μL of low glucose Ringer. The results using the ligated preparations of the caeca showed no greater response to the AST 5 than the flat-sheet preparations, and were therefore not used further.

Enzyme preparation and assays

The rate of enzyme release from the flat-sheet caecal preparations was almost constant over the tissue incubation time of 10–45 min for amylase, trypsin (Fig. 1a,b) aminopeptidase and carboxpetidase A (not shown). The 30-min tissue incubation time used was in the linear region. After incubation, the caeca were removed with minimal fluid loss from the incubation medium, and discarded. Thus, only the enzymes released during incubation were present in the supernatant, which was centrifuged for 5 min at 5000 ***g*** at 4 °C. The enzyme activity curves were also linear from 5–30 min for amylase and from 10–40 min for trypsin and aminopeptidase (Fig. 2). The incubation times used for the amylase, trypsin and aminopeptidase assays were again in these linear regions. The temperature and pH optima for the three enzymes studied along with their K_m and V_{max} values are given in Table 1.

The α-amylase activity was measured by adding 10 μL of the enzyme stock to 400 μL of 100 mM phosphate buffer, pH 7.2 containing 0.2 μM of the trypsin inhibitor *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) and the exoprotease inhibitor phenanthroline monohydrate (PAM) and starch (0.23 % end concentration). Incubation was performed at 30 °C for 30 min. The concentration of maltose released was determined using the dinitrosalicylic acid (DNS) method of Bernfeld (1955), whereby 400 μL of DNS reagent (1.6 g

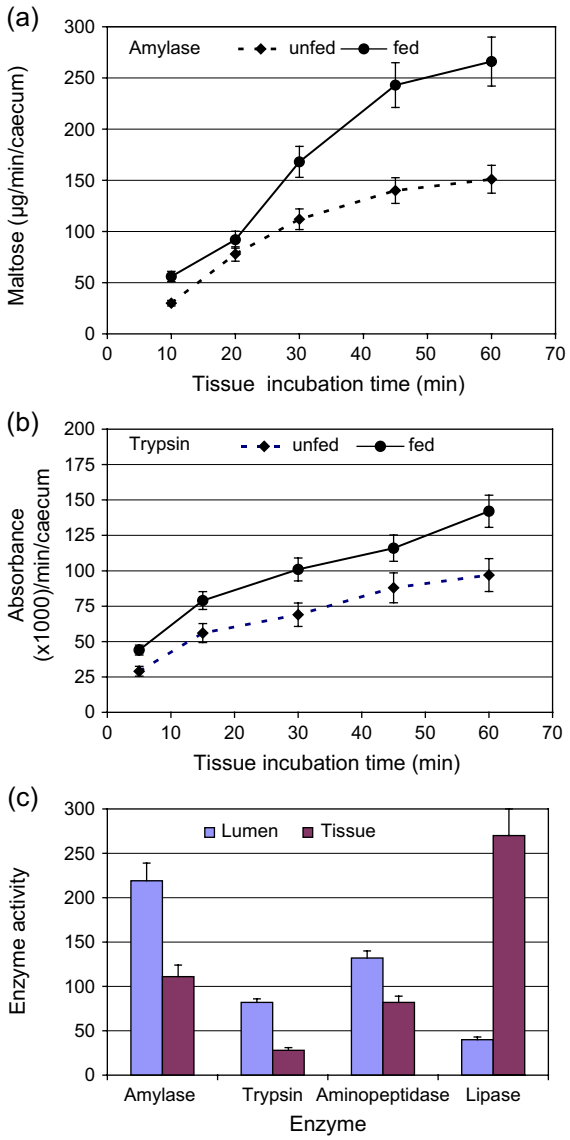

Fig. 1. (a) Amylase release in relation to caecum incubation time in low glucose Ringer at 30 °C for fed and unfed 2-day-old females ($n = 10$). (b) Trypsin release in relation to caecum incubation time ($n = 10$). (c) Comparison of enzyme activity released into the incubation medium (after a 30-min incubation) and in the tissue (cells) in 2-day-old fed females. All enzyme activities are given as units min^{-1} $caecum^{-1}$. Amylase activity units are µg maltose, trypsin units are change in absorbance (405 nm) × 1000, aminopeptidase change in absorbance (405 nm) × 100 and lipase activity units are nmol oleic acid ($n = 6$). Data are the mean ± SE.

NaOH + 30 g NaK-tartrate + 1 g DNS) was added to stop the reaction and the mixture heated to 100 °C for 10 min to develop the colour. After cooling in water, the maltose concentration was measured at 530 nm against a maltose standard curve. The 0.4 mg maltose added with the 10 µL of the enzyme (contained in the tissue incubation medium) to the 400 µL phosphate buffer of the enzyme assay yielded an

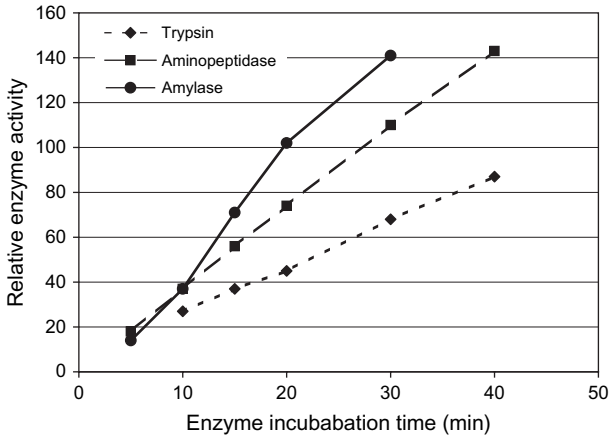

Fig. 2. Linearity of the relative enzyme activity in relation to the assay incubation time. The incubation time was 30 min for the amylase assay and 1 min for the trypsin and aminopeptidase assay (times 20 in this scale).

increase of only 10 µg maltose to the typical 200–300 µg maltose present in the amylase standard assay. To determine lipase activity, 50 µL of enzyme was added to 150 µL 100 mM phosphate buffer (containing the protease inhibitors TLCK and PAM; see above) plus 20 µL of 1 mM triolein (containing 100 mM Triton X-100) and incubated at 30 °C for 30 min. The liberated fatty acids were determined using the NEFA C test kit (Wako Chemical GmbH, Germany) where the absorbance (550 nm) was measured against an oleic acid standard curve. The trypsin activity was measured as the change in absorbance at 405 nm in 1 min at 25 °C after addition of 100 µL of enzyme stock to 700 µL of 100 mM phosphate buffer (pH 7.2) containing α-*N*-benzoyl-DL-arginine-*p*-nitroanilid (end concentration 0.87 mM). Leucine aminopeptidase was measured in the same manner as trypsin but with the substrate L-Leu-*p*-nitroanilid (end concentration 0.87 mM), and carboxypeptidase A activity with the substrate *N*-(3[2-furyl] acryloyl)-Phe-Phe (end concentration 0.087 mM).

Statistical analysis

The WinStat program for Excel (Microsoft Corp, Redmond, Washington) was used to evaluate the data. After determination of the normal distribution (*F*-test), the differences were tested with the Mann–Whitney *U*-test. Stastistical significance is shown in the graphs ($*P < 0.05$; $**P < 0.01$).

Results

The amount of all three enzymes released in the caecum of fed animals was consistently 20–70% more than in unfed animals but a considerable amount of enzymes was released even after 24 h without food (amylase and trypsin; Fig. 1a,b). In unfed animals feeding always stimulated within an hour an increase in enzyme release over the basal (unfed) levels,

Table 1. Temperature and pH optima of enzymes, K_m and V_{max} values.

Amylase	>35 °C	pH 6.2	K_m = 2.1 mg starch mL^{-1}	V_{max} = 2.8 μg min^{-1}
Trypsin	>35 °C	pH 8.1	K_m = 0.12 mM BApNA	V_{max} = 3.5 μmol min^{-1}
Aminopeptidase	>35 °C	pH > 8.1	K_m = 1.1 mM LpNA	V_{max} = 0.25 mmol min^{-1}
Lipase	37 °C	pH 8.0	ND	ND

BApNA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilid; LpNA, L-Leu-*p*-nitroanilid; ND, not determined.

which continued over the next 4 h (amylase; Fig. 3a). The released amylase, trypsin and aminopeptidase activity was double the enzyme activity within the caecal cells after only 30 min of incubation (Fig. 1c), whereas lipase was released hardly at all.

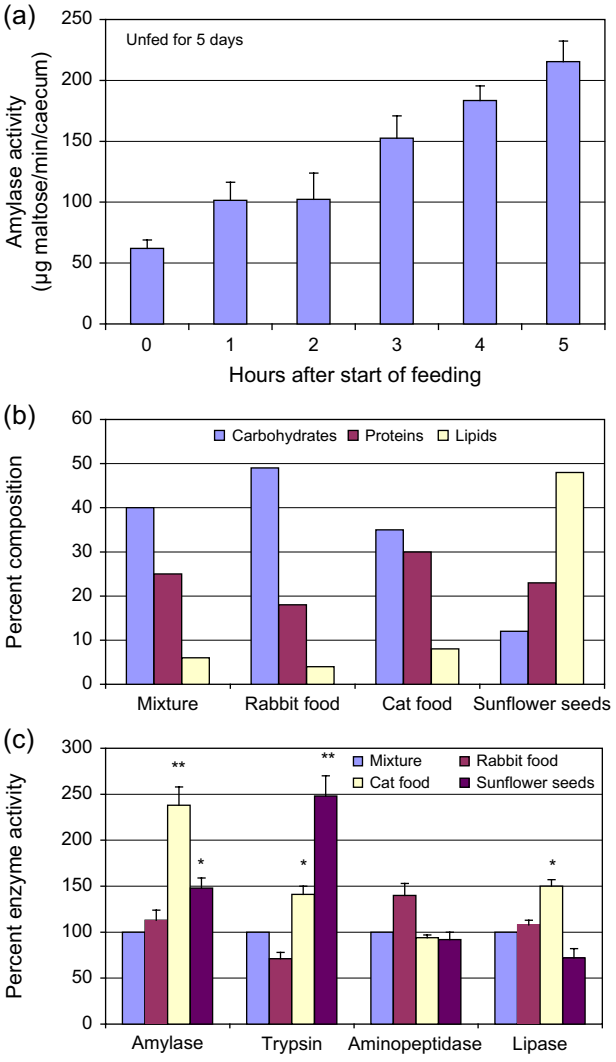

Fig. 3. (a) Increase of amylase release (μg maltose min^{-1} caecum^{-1}) in the caecum of 5-day-old unfed females 0–5 h after initiation of feeding with starch (n = 10). Data are the mean ± SE. (b) Composition of commercial diets (see Materials and methods). (c) Effect of feeding different diets on the percent change of enzymes released in the caecum (the Standard mixed diet was set to 100%) (n = 5). Data are the mean ± SE.

Not only was the enzyme release higher in animals fed on a mixed diet compared with unfed animals, but also the composition of the food had a profound effect on the ratio of the digestive enzymes released. Feeding stimulated the release of digestive enzymes in response to a specific ratio of nutrients (Fig. 3b), which led to a different ratio of digestive enzyme release (Fig. 3c). A high nutrient component in the food did not result necessarily in a correspondingly high digestive enzyme for that component. Unusually high levels of amylase were released in crickets fed cat food (high in protein) and unusually high levels of trypsin were released in crickets fed sunflower seeds (high in lipids).

There was no significant stimulation of amylase release in response to addition of starch to the incubation medium *in vitro*, but rather an unexpected inhibition ($P < 0.05$) at lower or moderate starch concentrations (Fig. 4a). The two sugars maltose and glucose stimulated amylase release significantly ($P < 0.05$ and $P < 0.01$, respectively; Fig. 4b,c), and there was no difference in the response of fed and unfed females to maltose (Fig. 4b). There was no effect of either starch or maltose on the release of any protease (results not shown). Stimulation of protease release in response to addition of proteins to the incubation medium occurred only with addition of 0.4–0.6 mg mL^{-1} of peptone for trypsin (Fig. 5b), a partial digest of casein, containing a mixture of short length polypeptides and amino acids. Surprisingly, after addition of high concentrations of both BSA and peptone, there was a general inhibition of trypsin activity in the incubation medium, but not of aminopeptidase and carboxypeptidase activity, (Fig. 5a,b). A mixture of 20 amino acids had no detectable effect on the trypsin or aminopeptidase release (Fig. 5c).

There was a stronger stimulation ($P < 0.01$) of the release of amylase *in vitro* in 2-day-old fed females in response to AST 5 compared with that of 1-day-old unfed females, but the trypsin release of unfed females was greater than that of the fed females (Fig. 6a,b).

Discussion

In *G. bimaculatus*, basal levels of protease, lipase and amylase are being released continuously even in unfed animals (Woodring *et al.*, 2007) and this is observed also for trypsin and aminopeptidase in the present study. The increased amylase release over several hours in starved crickets is observed also in *Periplaneta americana* (Sakai *et al.*, 2006).

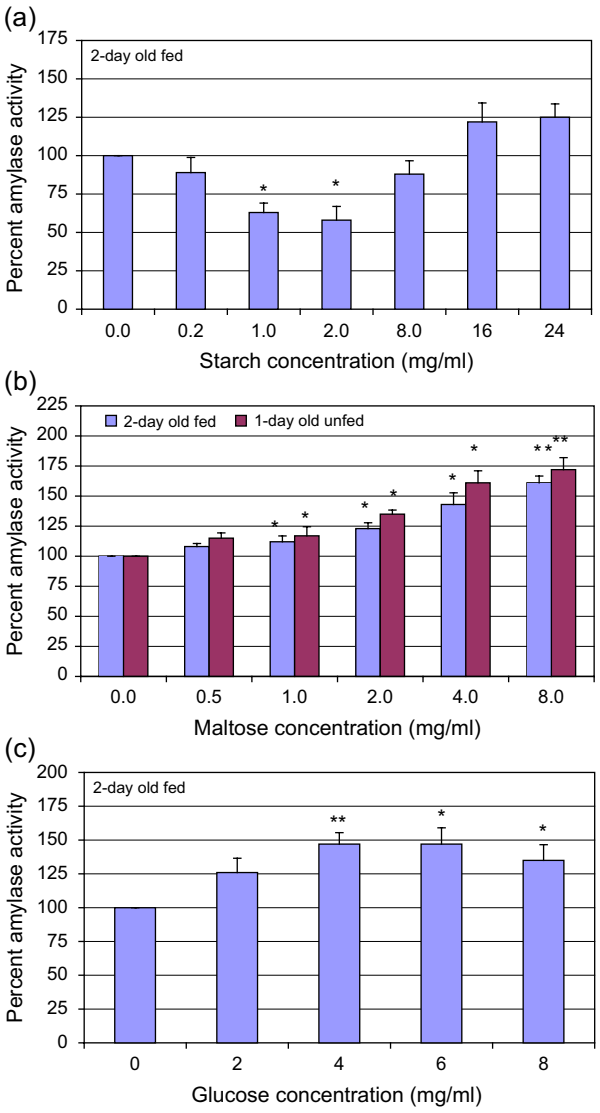

Fig. 4. The effect of (a) starch, (b) maltose and (c) glucose in the incubation medium (low glucose Ringer) on the *in vitro* release of amylase from flat-sheet caecal preparations after a 30-min incubation ($n = 10$ in all cases). Data are the mean ± SE.

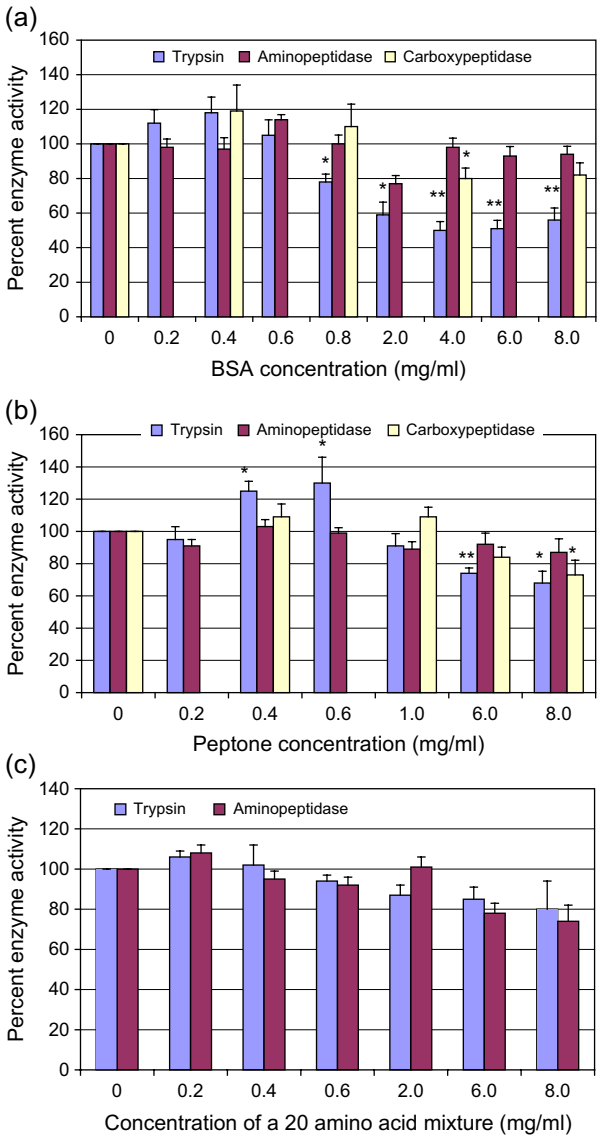

Fig. 5. The effect of (a) bovine serum albumin, (b) peptone and (c) a mixture of 20 amino acids in the incubation medium (low glucose Ringer) on the *in vitro* release of trypsin, aminopeptidase and carboxypeptidase A from flat-sheet caecal preparations of 2-day-old fed females ($n = 10$ in all cases). Data are the mean ± SE.

A minimal enzyme release is typical for many continuous feeders, particularly in orthopteroids (Chapman, 1985). The composition of the food likewise has a profound effect on the ratio of the digestive enzymes released. A variable ratio of nutrients leads to a different ratio of digestive enzyme release in *G. bimaculatus*. The adaptation of the enzyme activity in response to the ratio of nutrients in the gut is of distinct benefit to the insect and it is documented in a number of insects (Applebaum, 1985). However, the change in enzyme ratio in *G. bimaculatus* does not correspond to the expected change to specific diets. A form of compensation appears to occur, where the enzyme activity for the nutrient present in the lowest amount is elevated, at least in the case of the amylase. Crickets fed cat food and sunflower seeds (low carbohydrates) secrete more amylase than those fed on other diets, and crickets fed sunflower seeds (relative low protein content) secrete much more trypsin than those fed the other diets. The very high ratio of lipids in the sunflower seed diet do not elevate lipase release, but rather the excess lipids are simply egested, resulting in up to 35% lipids in the faeces (S. Diersch, unpublished results).

The cellular contents of caeca from unfed females have almost twice the amount of amylase activity compared with fed *G. bimaculatus* females (Woodring *et al.*, 2007), indicating that much of the amylase is not released until food is present. The increased enzyme release in less than 30 min means that

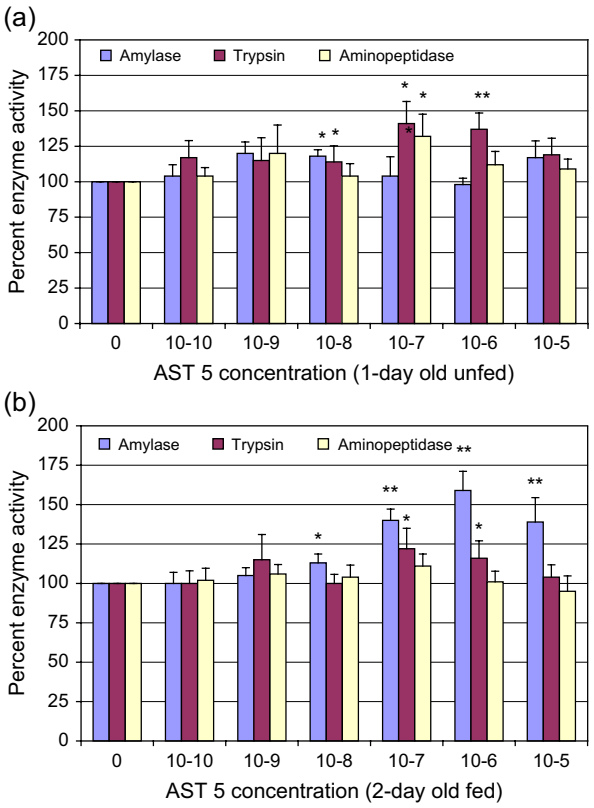

Fig. 6. The effect of the neuropeptide Grybi-AST 5 in the incubation medium (low glucose Ringer) on the release of amylase, trypsin and aminopeptidase *in vitro* from flat-sheet caecal preparation of (a) 1-day-old unfed females and (b) 2-day-old fed females ($n = 10$ in all cases). Data are the mean ± SE.

either the nutrient or neuropeptides stimulate release and not synthesis. The consistent amount of enzyme in the cells suggests that rate of synthesis and release are similar. These results confirm earlier reports in other insects, which suggest a level of enzyme synthesis paralleling the release rate (Chapman, 1985).

In vertebrates, the nutrients in the gut bind or interact with the lumen side of secretory cells, which induces a release of a peptide (cholecystokinin and gastrin) from cytoplasmic extensions on the blood side of the digestive epithelium. The peptides (a paracrine effect) are thought to act on nearby enzyme-producing cells. In insects, the endocrine cells are located between the epithelial cells of the midgut, and occur in all insects investigated (Lehane *et al.*, 1995). Long open-type endocrine cells, with the apical end exposed to the lumen and the expanded basal end containing numerous droplets and granules, occur in many insects, including *G. bimaculatus* (Sehnal & Zitnan, 1996).

A rinsed flat-sheet midgut preparation is a good way to demonstrate a paracrine mechanism in response to a particular nutrient. There is no evidence and no known mechanism in any animal in which nutrient receptors occur on the blood side of a gut that could stimulate gut endocrine cells (also on the blood side) to release a gut hormone. A nutrient can only bind to the lumen side of a cell, which means binding to the lumen end of an open type endocrine cell and inducing the endocrine cell to release a neuropeptide from the other end (Fusé *et al.*, 1999). It is assumed that only the lumen end of a paracrine cell in a flat-sheet preparation could have the appropriate receptors for hormone release. Binding of the nutrient element to the lumen side of the enzyme-producing cell (a prandial mechanism) cannot be excluded entirely, but appears to be unlikely.

Flat-sheet preparations can also be used to study (neuro)hormonal induced release of digestive enzymes, as in the present study in which it is assumed that receptors for the neurohomones are lacking on the lumen side. The neurohormone (AST 5 in this case) must bind to the blood (haemolymph) side of the enzyme-releasing cell to induce release of digestive enzymes into the lumen.

The chief component of the cricket standard diet is carbohydrate (40%) and so it would be expected that carbohydrates would have a greater influence on the release of digestive enzymes than other nutrients. Maltose and glucose increased amylase release, indicating the presence of maltose and glucose receptors on the lumen end of the gut endocrine cells. This sugar binding probably induces the release of paracrines on the hemolymph side, which then dock on adjacent or nearby enzyme-producing cells and induce amylase release. Unexpectedly, low or moderate starch concentrations causes an inhibition of amylase release below that of unfed controls. Amylase inhibitors are described in a number of insects that block the active site of the enzyme and do not affect amylase release (Franco *et al.*, 2002), which would explain the inhibition observed in *G. bimaculatus*. At higher concentrations, some starch is perhaps partially digested to maltose, which elevates amylase release and, thus, an apparent recovery from the inhibition is observed.

To obtain the necessary amino acids for growth and development, all organisms require protein and the associated proteases and, therefore, a stimulation of protease release in response to the presence of proteins would be expected. Indeed, the relatively high protein content of cat food fed to *G. bimaculatus* leads to a moderate stimulation of trypsin release. However, in the flat-sheet preparation, only peptone (a mixture of different length polypeptides and amino acids) elevates trypsin release. Thus, it would appear that some proteins (polypeptides) stimulate the release of the endopeptidase trypsin in the caecum but not the release of exopeptidases. In crickets, the continuous release of proteases is sufficient to meet the needs for growth, and only moderate stimulation of protease release results from feeding. In the blood-feeding fly *Stomoxys calcitrans*, all tested proteins stimulate the release for trypsin from the opaque zone of the midgut, but neither smaller polypetides nor mixtures of amino acids have any effect (Blakemore *et al.*, 1995).

An unexpected inhibition of the basal trypsin release rate occurs at higher concentrations of protein (BSA) in the incubation medium. The basal rate of trypsin release most likely does not involve paracrine release (e.g. in unfed animals). Excess binding of polypeptides to the endocrine cell would hardly lead to decreased trypsin release. The most

likely explanation for the inhibition of trypsin is a direct inhibition of the trypsin active site. Plant protein inhibitors are capable of forming stable complexes with the target proteases in the insect gut, blocking, altering or preventing access to the enzyme active site (Fan & Wu, 2005). Perhaps such 'excess substrate inhibition' occurs in *G. bimaculatus* where BSA irreversibly binds to the trypsin active site. Excess substrate inhibition occurs in approximately 20% of all enzymes (Birch, 2008).

Inhibition of enzyme release (not the actual enzyme activity) via various leucokinins is reported in the lepidopteran *Opisina arenosella* (Harshini *et al.*, 2002a). In cockroaches, allatostatins stimulate the release of carbohydrate digesting enzymes (Fusé *et al.*, 1999; Aguilar *et al.*, 2003). In *G. bimaculatus*, the allatostatin Grybi-AST 5 stimulates the release of amylase and trypsin. AST 5 is a paracrine secreted by the endocrine cells in the caecum, which is suggested by the expression of the AST 5 gene in the gut epithelium (Meyering-Vos & Hoffmann, 2003). A massive input of carbohydrates in fed females elevates amylase release strongly and this process appears to be supported by AST 5. The basal level of release of trypsin in fed females, however, appears sufficient, and an additional AST 5-mediated increased trypsin release occurs only at a low level. Underfed females, on the other hand, require amino acids to grow, and an AST 5 sensitivity to stimulate trypsin and aminopeptidase release, as observed in the present study, would be advantageous.

Acknowledgements

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Article

CONTROL OF THE RELEASE OF DIGESTIVE ENZYMES IN THE LARVAE OF THE FALL ARMYWORM, *Spodoptera frugiperda*

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There is a basal level of enzyme activity for trypsin, aminopeptidase, amylase, and lipase in the gut of unfed larval (L6) Spodoptera frugiperda. Trypsin activity does not decrease with non-feeding, possibly because of the low protein levels in plants along with high amino acid requirements for growth and storage (for later reproduction in adults). Therefore, trypsin must always be present so that only a minimal protein loss via egestion occurs. Larvae, however, adjust amylase activity to carbohydrate ingestion, and indeed amylase activity is five-fold higher in fed larvae compared to unfed larvae. Gut lipase activity is low, typical of insects with a high carbohydrate diet. A flat-sheet preparation of the ventriculus was used to measure the release of enzymes in response to specific nutrients and known brain/gut hormones in S. frugiperda. Sugars greatly increase (>300%) amylase release, but starch has no effect. Proteins and amino acids have little or no effect on trypsin or aminopeptidase release. The control of enzyme release in response to food is likely mediated through neurohormones. Indeed, an allatostatin (Spofr-AS A5) inhibits amylase and trypsin, and allatotropin (Manse-AT) stimulates amylase and trypsin release. Spofr-AS A5 also inhibits ileum myoactivity and Manse-AT stimulates myoactivity. The epithelial secretion rate of amylase and trypsin was about 20% of the amount of enzyme present in the ventricular lumen, which, considering the efficient

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counter-current recycling of enzymes, suggests that the secretion rate is adequate to replace egested enzymes. © *2009 Wiley Periodicals, Inc.*

Keywords: digestive enzymes; larvae; *Spodoptera frugiperda*

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is an important defoliator of corn, sorghum, cotton, and alfalfa in the tropical regions of the western hemisphere from the United States to Argentina. Various insecticides have been employed to control the fall armyworm, however, with only limited success and at a cost of considerable damage to the environment. The digestive tract of insects, especially caterpillars, is an excellent site for control mechanisms that in general are not toxic to other organisms. Endogenous enzyme inhibitors such as the trypsin-modulating oostatic factor (TMOF) effectively inhibit translation of trypsin mRNA in the larva gut of *Heliothis virescens* (Nauen et al., 2001). The crystal proteins (cry) are endotoxins produced by *Bacillus thuringiensis* (Bt), which punctures the insects' ventriculus and causes uncontrolled leakage and death in all lepidopteran larvae tested (Pigott et al., 2008). Control is very effective and specific, and the Bt gene has been inserted into many crop plants. There are a number of plant protease inhibitors (see review, Fan and Wu, 2005) that bind to endo- and ectopeptidases in insects and effectively inhibit growth, and likewise a number of plant α-amylase inhibitors (see review, Franco et al., 2002). Finally, in recent years it has been shown that members of several families of insect neuropeptides, including allatostatins (AS) and allatotropins (AT), control the release of digestive enzymes and at the same time modulate gut myoactivity in many insects (Fúse et al., 1999; Harshini et al., 2002a; Sakai et al., 2004; Hill and Orchard, 2005). Insertion of the genes of enzyme inhibitors or neurohormones into crop plants would be worth the effort, but only if more detailed information on the action of these peptides indicates effective in vivo regulation of digestive functions. The current investigation of the release of digestive enzymes in *S. frugiperda* is a step in this direction.

The proteases and amylases have been described in the gut of a number of noctuid larvae (Lenz et al., 1991; Johnson et al., 1995; Ortego et al., 1996; Bown et al., 1998), and the role of the peritrophic membrane and the counter-current flow of these enzymes in the ventriculus of *S. frugiperda* has been described in detail (Ferreira et al., 1994a,b; Bolognesi et al., 2001). In the current study, the distribution of lipase in addition to trypsin and amylase in the gut, the effect of larval age, and especially the role of feeding on enzyme release in *S. frugiperda* are examined. The main thrust of the current study however, is an examination of the effect of specific nutrients and neurohormones on the release of enzymes from incubated midguts (flat-sheet preparations), as previously described in *Gryllus bimaculatus* (Woodring et al., 2009). The neurohormones tested are based on the gene sequences of two allatostatins (Spofr-AS A5, AS A6) and an allatotropin (Manse-AT) found in *S. frugiperda* (Abdel-latief et al., 2004). Many neurohormones that affect enzyme release also affect gut myoactivity (Harshini et al., 2002b; Matthews et al., 2007), and, therefore, the myoactivity of these two AS and an AT is also examined.

MATERIALS AND METHODS

Rearing Methods and Diets

Pupae and eggs of *S. frugiperda* and an artificial diet (based on bean meal) were provided by Bayer CropScience AG (Monheim). Larvae were reared at 27°C and approximately 70% relative humidity under a L16:D8 photoperiod on the Bayer diet, as previously described (Oeh et al., 2000). To prevent cannibalism, L4 larvae were maintained individually in separate compartments of assortment boxes with 40 compartments (49 × 32 × 36 mm per compartment). The pupae were also separated, and after emergence a 2:1 ratio of males to females were placed in a 30 × 30 × 15-cm container with a sucrose solution as a food source, water, and paper as a substrate for egg laying (Oeh et al., 2000). In order to test the effect of diet and feeding on enzyme activity, some newly moulted L6 were provided a cellulose diet (30 g cellulose powder +3 g agar in 250 ml water) and others received no food at all. Unless otherwise stated, the L6 day-3 larvae were used in all experiments.

Gut Preparation and Incubation

The midgut (ventriculus) averaged 21 mm in length, and the hindgut (Ileum+colon) averaged 5–6 mm. These were separated, and the enzyme activity was measured in the tissues and in the lumen. The ventricular lumen included the exo- and endoperitrophic space plus the peritrophic membrane (PM). The total lumen contents were brought to 300 µl with *Spodoptera* Ringer (per Liter 0.7 g NaCl, 2.0 g KCl, 0.25 g $NaHCO_3 \times 2H_2O$, 1.3 g $CaCl_2 \times 2H_2O$, 4.4 g $MgCl_2 \times 7H_2O$, 1.0 g Hepes at pH 7.0). The tissue was sonicated lightly (BronsonSonifier 250, for 2–3 sec at the lowest setting at 4°C) and centrifuged at 12,000*g* (4°C) for 10 min. The secretion of enzymes was determined using flat-sheet preparations. Freshly dissected ventriculus were slit open and rinsed twice with fresh low glucose Ringer (LGR) (10 mg glucose/100 ml Ringer), a concentration adapted from Fúse et al. (1999) for incubation of gut tissues of the cockroach *Diploptera punctata*, placed in 300 µl LGR, and incubated for 30 min at 30°C without shaking. The tissue was discarded, and the remaining incubation medium was centrifuged and used to measure enzyme release. Thus, there were three sources of enzymes: lumen content, tissue (cells) homogenate, and incubation medium. The released amylase, lipase, trypsin, and aminopeptidase each increased at a constant rate over an incubation time from 10 to 30 min (Fig. 1), indicating that the tissues were viable over the test period.

Enzyme Assays

For the determination of enzyme activity, 10 µl of the homogenate or incubate was used for the amylase assay, 50 µl for the lipase assay, and 100 µl for the trypsin and aminopeptidase assay. It was found that the amylase and lipase release (increase in activity) was linear from 15 to 60 min, and the trypsin and aminopeptidase activities were linear from 0 to 5 min at 25°C and at a pH of 8.0 (data not shown). For the amylase assay, 10 µl of the homogenate or incubate was added to 40 µl 2.5% starch+ 360 µl Tris buffer (652 ml 0.1M Tris+348 ml 0.1M HCl) and incubated 30 min at 30°C with light shaking. To this mixture, 400 µl dinitrosalicylic acid Reagent (1 g dinitrosalicylic acid in 70 ml 2N NaOH, 30 g KNa tartrate, with water made to

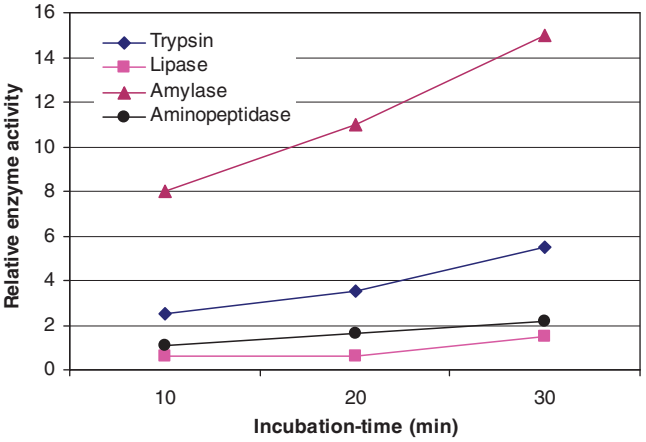

Figure 1. The rate of digestive enzyme release from the flat-sheet preparation of the ventriculus incubated in low glucose ringer (10 mg glucose/100 ml *Spodoptera* Ringer) for 30 min.

100 ml) was added, heated to 100°C for 10 min and cooled to room temperature with water.

A blank (zero) and maltose standard (100 µg maltose), both with 0.1 mg starch (0.23%), was run with every sample tested, and therefore any maltose associated with the added substrate (starch) was cancelled out. For the lipase assay, 20 nmol triolein (from a stock solution of 10 mM triolein+1,300 mM Triton × 100 (= 44 mg triolein+ 4 g Triton in 5 ml EtOH) was evaporated dry at 40°C in a 1.5-ml Eppendorf tube, and 150 µl Tris buffer (above) was added along with 50 µl of the homogenate or incubate. After 30-min incubation at 30°C with light shaking, 200 µl of reagent A (Fatty Acid Kit, Wako, Neuß, Gemany) was added and incubated 10 min at 30°C with shaking, followed by 400 µl of reagent B also incubated 10 min at 30°C with shaking. A standard curve was constructed with 1 nmol oleic acid at 550 nm, and the amount of oleic acid produced was measured. For both the amylase and lipase assays, 0.1 µM of the trypsin inhibitor N-α-tosyl-L-lysine chloromethyl ketone (TLCK) and 0.1 mM of the exopeptidase inhibitor phenanthroline monohydrate (PAM) was included in the incubation mixture. For the trypsin and aminopeptide assays, the release of NA (dAbs/ min) from the synthetic substrates α-N-benzoyl-DL-arginine-p-nitroanilid (BApNA) and L-leucine-p-nitroanilid (LpNA), respectively, was measured at 405 nm. Possible leakage of aminopeptidase from cells during ventricular incubation was estimated by measuring leakage of the intracellular enzyme acid-phosphatase into the incubation medium. Only 0.034 ± 0.003 ($n = 10$) units acid-phosphatate/min was released from 1 mM of the substrate p-nitrophenyl phosphate after 30-min incubation of the tissue.

The Km, Vmax, optimum pH (0.1M Tris/HCl), and optimum temperature for each enzyme are listed in Table 1. One amylase unit is the amount required to liberate 1 µg maltose qeuivilents from starch/min, one trypsin (or aminopeptidase) unit is the amount required to liberate 1 µmol BApNA (or LpNA, respectively)/min. One lipase unit is the amount required to liberate 1 nmol oleic acid from triolein.

Nutrients and Neuropeptides Tested

The effect of nutrients or neuropeptides on the rate of enzyme release was determined by adding the test material to 300 µl of LGR incubation medium. The carbohydrates

Table 1. Enzyme Kinetics

Enzyme	*Km*	*Vmax/ventriculus*	*Opt pH*	*Opt Temp*
Amylase	0.29% Starch	59 µg Maltose/min	ca 9.0	>40°C
Lipase	0.30 nM Triolein	25 nmol Oleic acid/min	ca 9.0	>40°C
Trypsin	0.93 mM BapNA	3.0 µmol/min	ca 9.0	>40°C
Aminopeptidase	0.23 mM LpNA	10.5 µmol/min	ca 9.0	>40°C
BapNA-α-N-benzoyl-DL-arginine-p-NA		LpNA- L-leucine-p-NA		

tested were 0.1 to 2.0 mg/ml (final concentration) of starch, maltose, and glucose. The protein tested was 0.1 to 2.0 mg/ml of bovine serum albumin (BSA). Peptone (a mixture of small peptides) and a mixture of all 20 amino acids was also tested. Two allatostatins were tested, 10^{-8} to 10^{-5} M Spofr-AS A5 (ARAYDFGLa) and 10^{-8} to 10^{-5} M Spofr-AS A6 (LPMYNFGLa). The two AS were custom synthesized by Biosyntan GmbH, Berlin. A concentration of the allatotropin Manse-AT (GFKNVEMMTARGFa) (from Bachem, Weil am Rhein, Germany) was tested from 10^{-8} to 10^{-5} M.

Assay of Myoactivity

In order to measure the effect of allatostatin and allatotropin on the myoactivity of the ileum, the hindgut with a short piece of attached ventriculus at the anterior end and colon-rectum at the other end (Fig. 2) was dissected out and placed immediately in a 200-µl drop of high glucose Ringer (HGR) (4 g glucose/100 ml *Spodoptera* Ringer), a mixture adapted from Ai et al. (1995) for neuromuscular transmission in larval Lepidoptera, on a plastic surface (Petri dish) at room temperature. Only moderately filled ileums with an undamaged section of attached ventriculus, from which the peritrophic membrane with gut contents could be removed, showed a reliable contraction rate and only these were used. The isolated ileums seldom started to beat earlier than 10 min in the HGR and sometimes only after 30 min. Typically, an ileum would start to beat and continue for about 5 min, but often it would beat for 20 min or more, and then stop. The inhibitory peptides *S. frugiperda* allatostatins (AS) were tested with such spontaneously beating ileums (typically 20–25 contractions/min) in 100 µl HGR, to which 10 µl AS was added. The reduced beat rate/min was counted, and a complete inhibition resulted in complete cessation (0 beats/min). The ileum was then returned to fresh HGR. If the original control rate (20–25 beats/min) was not observed, the test was aborted. Inhibition was recorded only when the same beat rate was observed with at least two transfers, and with at least 3 different ileums. The control of the contraction rate for inhibition in HGR was set to 100%, and the contraction rate with added neuropeptide was converted to % change (Table 2). The stimulating neuropeptide *M. sexta* allatotropin (Manse-AT), on the other hand, was tested only with non-contracting ileums (0 beats/min), whereby 10 µl of the AT was added to the 100-µl HGR. The contraction rate was measured for 1 min, then the ileum was transferred to a fresh 100-µl drop of HGR and kept there until a control 0 beats/min was again observed (usually in < 2 min). The results were usually consistent with up to 3–4 transfers back and forth from HGR and HGR+ allatotropin.

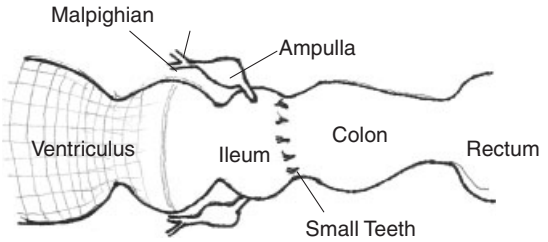

Figure 2. Schematic diagram of the hindgut of *Spodoptera frugiperda*.

Table 2. Effect of Neuropeptides on the Contraction of the Ileum of S. frugiperda

Neuropeptide	*Stimulatory*	*Inhibitory*
Proctolin	10^{-4} to 10^{-6} M	Not tested
Manse-AT	10^{-4} to 10^{-5} M	Not tested
Spofr-AS 5	No effect	10^{-5} to 10^{-8} M
Spofr-AS 6	No effect	10^{-5} to 10^{-8} M

RESULTS

Effect of Age and Feeding

Larval age, the gut region and site (lumen or cells), and feeding (or non-feeding) all influenced digestive enzyme activity. The amount of enzyme (activity) released from the ventricular cells during the last larvae stadium (L6) increased from day 1 to a maximal activity on day 3, and then dramatically decreased on day 4 (prepupae) as the digestive tract emptied and started to disintegrate (Fig. 3). The total activities of all four enzymes studied were much higher in the ventriculus than in the ileum, because the ventriculus is about 10 times larger than the ileum. Aminopeptidase was much higher in the cell fraction and amylase and trypsin much higher in the lumen (Fig. 4). The effect of a nutritive diet (Bayer CropScience AG), compared to not being fed or being fed a non-nutrient diet (cellulose), upon the enzyme activity in the ventriculus varied with the site and with the enzyme (Fig. 5). A doubling of lipase and aminopeptidase activity in response to diet-feeding was seen in the cellular fraction of the ventriculus, and a five-fold increased amylase activity in response to feeding was seen in the lumen contents. Feeding had no effect on enzyme activity in all other cases, which was particularly interesting regarding the constant trypsin activity in the lumen. An important observation was that there was a consistent basal level of enzymes secreted, activity in the lumen and in the cellular fraction in unfed (or cellulose-fed) larvae.

Enzyme Activity

Whether the enzyme release rate was sufficient to maintain the standing amount present in the lumen was determined in the following manner. The time of food passage through the ventriculus, based on feeding L6 larvae carmine red–colored diet ($n = 4$), averaged 3 h (= 180 min). The standing amylase activity in the ventricular lumen was 340 μg maltose/min, and the secreted activity of the incubated flat-sheet

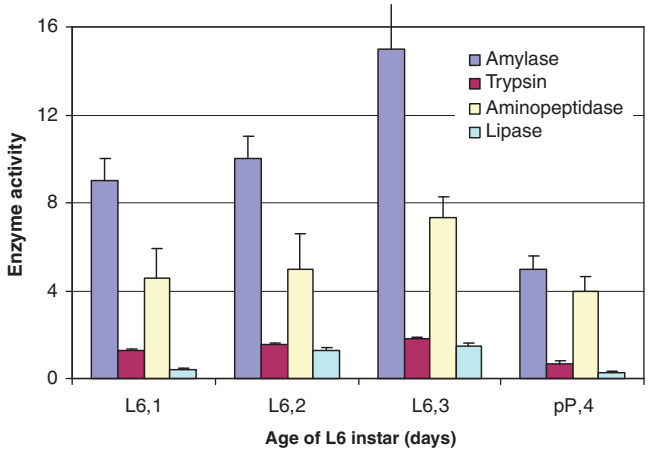

Figure 3. The release of amylase, lipase, aminopeptidase, and trypsin from the ventriculus of different aged L6 larvae (days 1–3) fed an artificial diet (Bayer CropScience, Monheim). The lipase values are multiplied by 10 (only in this figure) for ease of comparison. Units: see Materials and Methods.

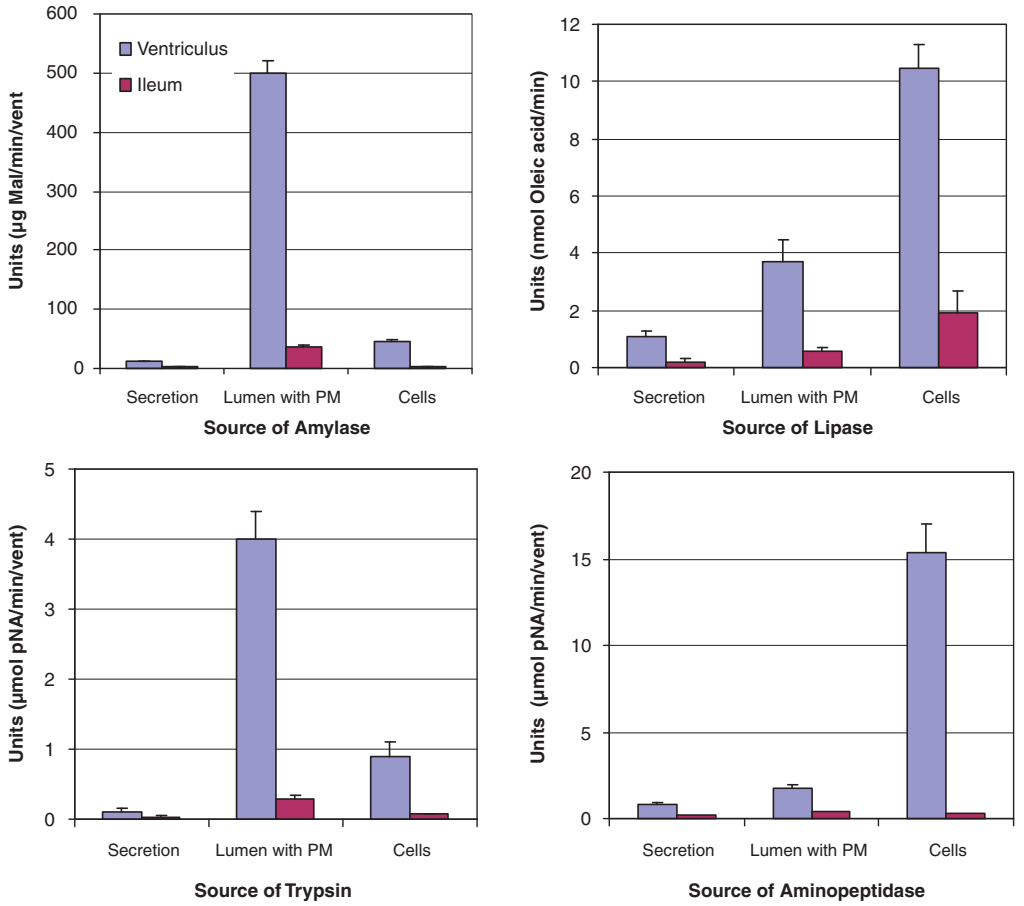

Figure 4. Amylase, lipase, trypsin, and aminopeptidase release from crop, ventriculus, and ileum of L6, day-3 larvae-fed synthetic diet.

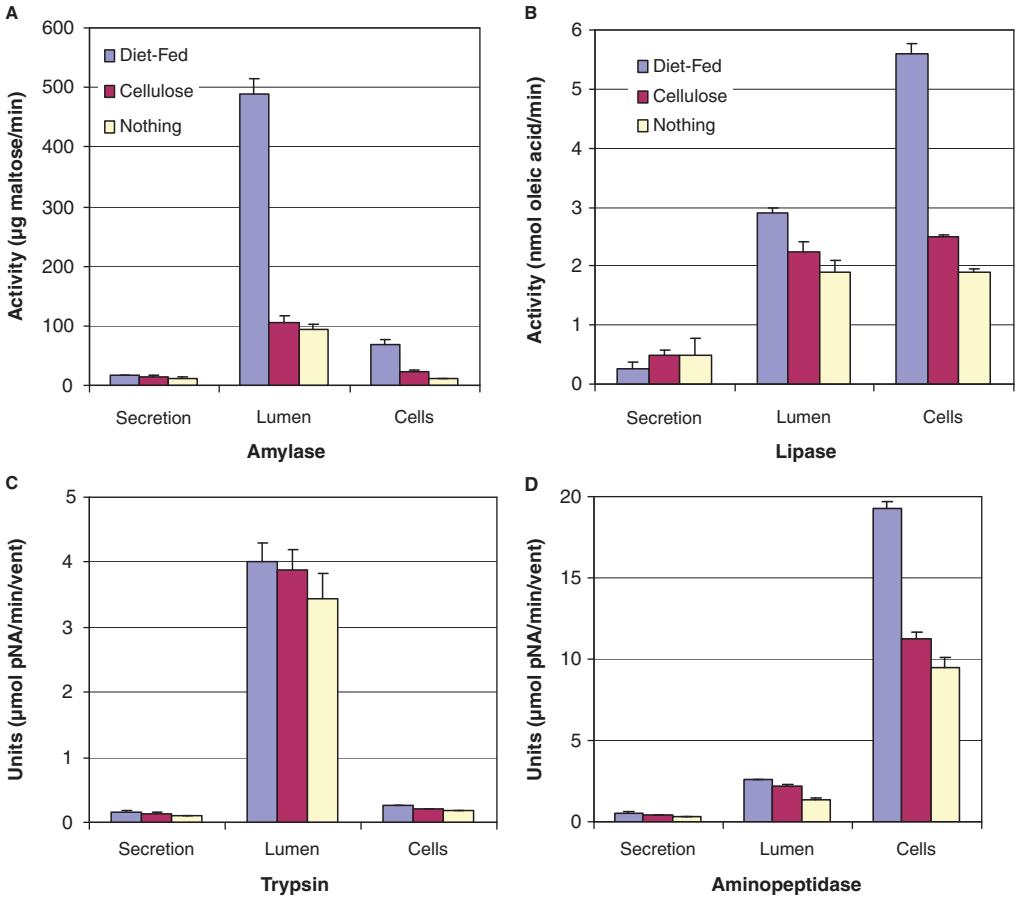

Figure 5. Amylase (**A**), lipase (**B**), trypsin (**C**), and aminopeptidase (**D**) release from the ventriculus of L6, day-3 larvae provided a synthetic diet (see Fig. 3), a cellulose diet, or nothing immediately after the moult to L6.

prepared ventriculus was 12 µg maltose/min after 30 min incubation (Fig 4). Therefore, in 3 h (= 180 min) 72 µg maltose/min was secreted, which was about 20% of the standing amylase activity in the lumen. The standing trypsin activity in the ventriculus was 3.8 units/min, and the secreted activity was 0.11 units/min after 30-min incubation. In 3 h, 0.66 units/min was secreted, which was about 17% of the standing trypsin activity. The standing lipase activity was 3.7 nmol/min over a 30-min incubation. In 3 h, 7.8 nmol/min lipase activity was secreted, which was more than the standing lipase activity. The standing aminopeptidase in the lumen was only 1.8 µmol/min. The very small aminopeptidase activity found in the incubate (Fig. 4) was probably released from damaged cells, not from secretion.

The ratio of the amount of enzyme (activity) in the ventriculur lumen relative to the amount in the ileal lumen was examined in order to estimate enzyme loss via egestion . There was 10 times more amylase and trypsin in the lumen of the ventriculus than in the ileum, and only 6 times more lipase in the ventriculus as in the ileum (Fig. 4). Over 2/3 of the lipase activity and over 90% of the aminopeptidase activity was associated with the tissue extract.

Effect of Specific Nutrients on Digestive Enzyme Release

To more precisely determine the role of feeding on the increased secretion of enzymes over that of basal levels, specific nutrients were added to incubated flat-sheet preparations of the ventriculus. A concentration of 0.5 mg/ml or higher of maltose or glucose in the incubation medium resulted in an up to 350% elevation of amylase secretion (Fig. 6A). The maximum effect ($P < 0.05$) of starch on amylase release was a modest 30–40% at > 0.5 mg/ml. The protein BSA had no effect on trypsin (Fig. 6B), and peptone (a mixture of small polypeptides) caused only a moderate increase ($P < 0.05$) in trypsin.but only at a concentration of 1.0 mg/ml (Fig. 6B). The effect of nutrients on the release of lipases was not studied because of the very low activities. The aminopeptidase activity in the incubation did not result from secretion, but rather from broken cells.

Effect of Neurohormones on Digestive Enzymes Release

Based on the identification of the gene sequence for Manse-AT and Spofr AS A5 and AS A6 in *S. frugiperda* (Abdel-latief et al., 2004), the effect of these neuropeptides on the release of amylase, trypsin, and aminopeptidase was investigated. The allatostatin

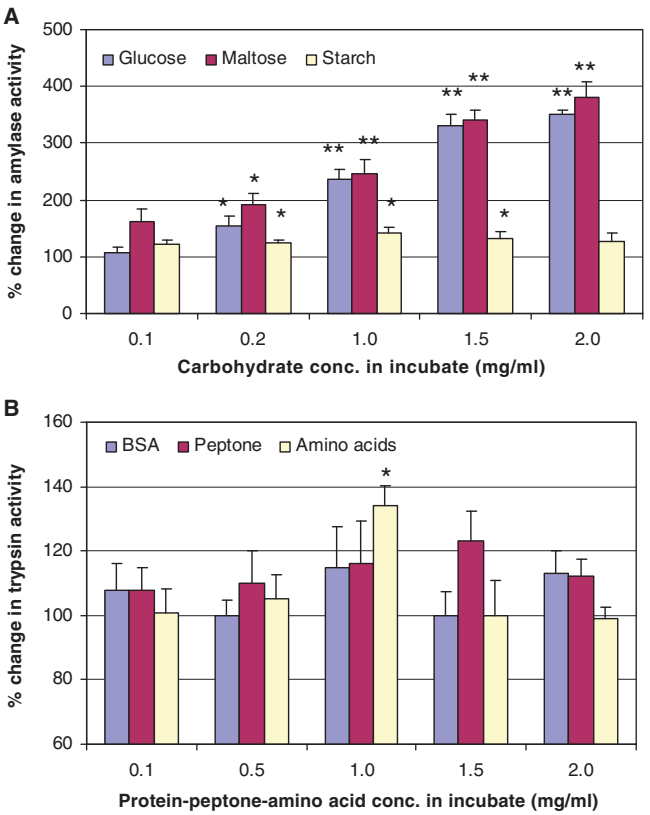

Figure 6. Effect of nutrients in the incubation medium of the ventriculus (L6, day 3) on the enzyme release (30-min incubation at 30°C). **A:** Effect of glucose, maltose, and starch on amylase release. **B:** Effect of BSA, peptone, and an amino acid mix on trypsin release. Control Ringer = 100%. Significant increase: $^{*} > 0.05$, $^{**} > 0.01$.

Spofr-AS A5 inhibited the in vitro release of amylase and trypsin by the ventriculus at concentrations of 10^{-5} and 10^{-6} M, but not at 10^{-7} or less (Fig. 7A). Spofr-AS A6 had no effect at any concentration (not shown). The allatotropin Manse-AT significantly stimulated amylase release at concentrations from 10^{-5} to 10^{-6} M but not at 10^{-7} M or less, and stimulated trypsin release from 10^{-5} to 10^{-7} M but not at 10^{-8} M or less. None of the tested AS or AT had any effect on aminopeptidases (Fig. 7).

Effect of Neurohormones on Gut Myoactivity

Increased contraction of ileal muscles could only be observed in a resting ileum with no spontaneous contractions, that is, 0 beats/min (Table 2). Relatively high concentrations (10^{-6} M to 10^{-4} M) of proctolin were required to stimulate the contraction of resting isolated ileums. Concentrations of Manse-AT from 10^{-5} to 10^{-4} M, but not at 10^{-6} M, also elevated the contractions from 0 beats/min to over 20 beats/min. Interestingly, the recovery back to 0 beats/min was gradual and required several transfers to fresh HGR over 3–4 min. Ileums that started a spontaneous contraction were basically not affected by application of Manse-AT. Neither of the two AS tested showed any myostimulatory effects. Inhibition of the ileal contraction rhythm could only be observed in an ileum that started to beat spontaneously. The spontaneous rate ranged from 15 to 30 beats/min, but once started the rate was constant for any single ileum over a 5–20-min period. Therefore, the amount of inhibition as a percent

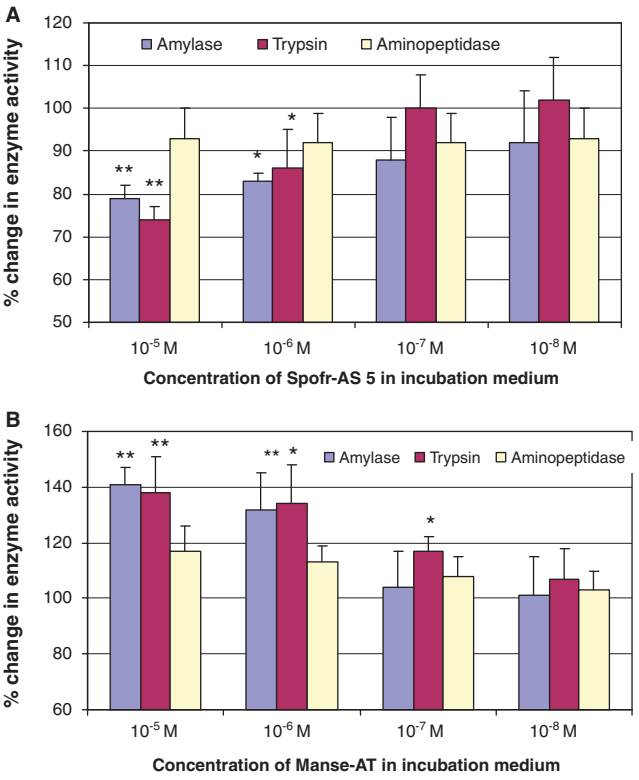

Figure 7. Effects of neuropeptides added to the incubation medium of the ventriculus (L5, day 3) on the enzyme release (30-min incubation at 30°C). **A:** Effect of the allatostatin Spofr-AS A5. **B:** Effect of the allatotropin Manse-AT. Control Ringer = 100%. Significant increase or decrease: * > 0.05, ** > 0.01.

change from the control (initial rate) in this time span was consistent and repeatable. The ileum was very sensitive to inhibition by both AS (Spofr-AS A5 and AS A6) at all concentrations over 10^{-8} M, showing an immediate complete inhibition (0 beats/min). At 10^{-9} M, the inhibition averaged 50% of the control, and there was no inhibition at 10^{-10} M. Recovery in fresh HGR required 20–30 seconds.

DISCUSSION

The ventricular lumen volume is about 150 µl and the ileum only about 10 µl, therefore the ileum should contain 15% of the total amount (activity) of luminal amylase and trypsin. However, the ileum activity is about half (7%) of that of the ventricular lumen. The lower concentration of amylase and trypsin in the ileum compared to the ventriculas suggests that the efficient counter-current flow of enzymes in the ventriculus of *S. frugiperda*, as described by Bolognesi et al. (2001), reduces the loss of important digestive enzymes via egestion.

As in other lepidopteran larvae (Ortego et al., 1996; Lenz et al., 1991), trypsin and amylase in *S. frugiperda* occur primarily in the endoperitrophic space and the aminopeptidase is bound to the ventricular cell membranes. Amylase and trypsin are derived from membrane-bound forms, and are released in a soluble form by a microapocrine mechanism into the ectoperitrophic space and diffused into the endoperitrophic space (Ferreira et al., 1994b). Part of the amylase (13%) and trypsin (18%) remains membrane-bound and some is incorporated into the peritrophic membrane (PM) (Ferreira et al., 1994a). That almost all trypsin and amylase activity in the current study is found in the ventricular lumen, and only about 20% in the cellular fraction, corroborates that earlier study. The virtual lack of aminopeptidase in the incubation medium and the low levels in the ventricular incubation medium found in the current study likewise confirms the earlier report (Ferreira et al., 1994b), in which small amounts of aminopeptidase bound to membranes in the ectoperitropic space and incorporated into the PM was found. That a significant amount of a soluble lipase occurs in the lumen of *S. frugiperda* has not been previously reported. The pattern of the distribution of trypsin, aminopeptidase, and amylase reported here is completely compatible with the model of the counter-current flow of digestive enzymes previously described (Terra et al., 1979; Ferreira et al., 1994b; Bolognesi et al., 2001). Most of the initial digestion (amylase and trypsin) occurs in the endoperitrophic space, and intermediate and final digestion (maltase, aminopeptidase, and carboxypeptidase) occur in the ectoperitrophic space or at the surface of the midgut cells.

The total lipase activity of *S. frugiperda* is low, but the lumen fraction of the total is relatively high. It was not possible with the methods employed to determine if the lipids in the cellular fraction are part of the luminal membrane or intercellular, but in any case, a large portion of lipid digestion seems to occur in the lumen. Based on studies with radiolabelled triolein, dietary triacylglycerol in *Manduca sexta* larvae is completely hydrolyzed to free fatty acids in the lumen before absorption into the cells (Tsuchida and Wells, 1988). Very little is known of the flow of lipase in the gut of insects. Amylase is secreted at the anterior end of the ventriculus and passes into the endoperitrophic space (Ferreira et al., 1994b). Lipase and amylase have similar molecular weights (50–60 kD) and it would be expected that soluble lipase also passes into the endoperitrophic space at the anterior end of the ventriculus.

Table 3. Relative Lipase-Amylase Activity in Selected Insects

Species	*Relative midgut weight (mg)*	*Amylase activity (μg maltose/min)*	*Lipase activity (μg oleic acid/min)*	*Ratio*
Lipase/amylase				
G. bimaculatus[a]	10	1200	28	0.015
S. frugiperda	1	220	2.0	0.011
O. fasciatus[b]	0.1	4	1.6	0.4

[a]Woodring et al. (2007a).
[b]Woodring et al. (2007b).

The lipase/amylase ratio can indicate the adaptation of an insect to its diet. The lipase activity (lumen plus cells) in *S. frugiperda* is 2.5 μg oleic acid/ventriculus/min (9 nmol), in *G. bimaculatus* 28 μg/caecum/min, and in *Oncopeltus fasciatus* 1.6 μg/ventriculus/min (Table 3). These three insects are quite different in size. The midgut of *G. bimaculatus* is about 10 times heavier than that of *S. frugiperda*, which is about 10 times heavier than that of *O. fasciatus*. The total activity is clearly related to size. However, making a ratio of the total lipase activity (μg oleic acid/min) to amylase activity (μg maltose/min) eliminates the size effect. The ratio in the cricket and the army worm are quite similar, and about 20 time less than that of the milkweed bug. The cricket *G. bimaculatus* is a detritus-feeder (mostly carbohydrates), *S. frugiperda* is a plant-feeder (also mostly carbohydrates), and *O. fasciatus* is a seed feeder (very high in lipids).

An interesting and important question is whether the amount of enzyme (activity) released (secretion) from the tissue is sufficient to replace that lost through egestion. The secretion rate of amylase and trypsin is about 20% of the standing enzyme activity in the lumen. With this low secretion rate, very little amylase or trypsin can be egested without reduction of the standing activity in the lumen. Perhaps the *in vitro* secretion rates obtained are lower than the actual in vivo secretion rates. If so, then this, along with the low egestion rates (ration of ventriculs/hindgut enzyme activities), would indicate that the secretion of enzymes balances egestion losses.

Regulated secretion of digestive enzymes in insects, in which enzymes are synthesized and stored and then released in response to an appropriate signal, only occurs in some (but not all) intermittent feeders (such as mosquitoes) (Lehane et al., 1996). In continuously feeding insects, there are few benefits to be gained from regulated rather than constitutive secretion. A basal release rate of enzymes into the ventriculus independent of the presence of food in the gut is found in many insects (Chapman, 1985); however, feeding almost always leads to an increase in the release and amount of enzymes in the gut (Applebaum, 1985; Woodring et al., 2009). In the case of *S. frugiperda,* the basal (minimal) release of amylase, trypsin, aminopeptidase, and lipase is the same for larvae fed a non-nutrient cellulose diet and those not fed at all, but feeding a nutritive diet results in increased enzyme secretion, which means nutrient composition and not merely bulk in the ventriculus is responsible for the increased enzyme release over that of the basal level.

In the cockroach *Leucophaea maderae,* feeding stimulated the production of proteases in the midgut (Engelmann and Geraerts, 1980). The response to feeding or not feeding on the release of amylase and trypsin in *S. frugiperda* is quite different. The amylase release is three times higher in fed than in unfed larvae, but trypsin release is

the same for both fed and unfed larvae. Both enzymes are immediately released after synthesis into the lumen (Ferreira et al., 1994a,b), which means that the synthesis rate must be related to nutrients in the gut. But why does amylase release vary with the feeding and trypsin release does not? A rationale based on food utilization can be hypothesized. The primary nutrient in the diet of the caterpillar is carbohydrate, much of which is converted to lipids and stored in the fat body. The adult moth subsequently uses the lipid as a flight fuel (Wheeler, 1989). Reduced dietary carbohydrate simply means less lipid reserves, and it would be wasteful to produce large amounts of amylase when food is in short supply. Therefore, the larvae can afford to adjust the amylase release to the carbohydrate intake. Proteins, on the other hand, are essential for larval growth, and egg production in the adult moth depends entirely on proteins stored in larval fat body (Sorge et al., 2000). The caterpillar, therefore, can not afford to allow any dietary protein to pass undigested through the gut. Therefore, it is advantageous to maintain a constant level of trypsin release in the event that even a small amount of protein is ingested. The release of aminopeptidase is twice as high in fed than in unfed larvae, which indicates some adjustment to gut peptide levels.

In order to more precisely determine the role of feeding on gut enzymes, the increased enzyme secretion over basal levels in response to the addition of specific nutrients to incubated flat-sheet preparations (*in vitro*) of the ventriculus were carried out. The larvae of the fall armyworm are plant-feeders, and simple sugars and polysaccharide intake is therefore high. In the current study, glucose and maltose provide a strong signal to release amylase, which is the normal way to stimulate amylase release in all animals. There is no evidence in vertebrates or insects of starch or other polysaccharide receptors on gut neuroendocrine cells that induce amylase release. The presence of starch in the incubation medium, however, appears to only mildly stimulate amylase release in *S. frugiperda*. This anomaly is most likely due to a minimum digestion to maltose or a small amount of sugar in the starch tested, and it is the maltose or glucose that stimulates amylase release and not the starch.

Similar to other insects, sugars bind to receptors in the apical membrane of neuroendocrine cells in the epithelial layer of the ventriculus, and induce the release of neuropeptides at the basal end into the hemolymph. A binding of sugars to the apical (lumen) end of the gut neuroendocrine cells is postulated in *G. bimaculatus,* which induces the release of neuropeptides from the basel end into the hemolymph (Woodring et al., 2009). Specific neuroendocrine cells in the gut epithelium of cockroaches and locusts release several kinds of neuropeptides that stimulate the release of amylase (Fúse et al., 1999; Sakai et al., 2004; Hill and Orchard, 2005). The in vitro inhibition or stimulation caused by allatostatin and allatotropin, respectively, in the current study also indicates that the final step in the regulation of amylase release in response to sugars is mediated by neuropeptides. The trypsin release is not elevated by feeding in *S. frugiperda*. Therefore, it is not surprising that bovine serum albumin (BSA) has no effect on in vitro release, and that peptone (a mixture of different sized peptides) or a mixture of all 20 amino acids has only a weak effect on the in vitro release of trypsin in the incubated flat-sheet preparations. BSA in the bloodsucking fly (*Stomoxys*), on the other hand, has a strong stimulating effect on the release of trypsin (Blakemore et al., 1995), which makes sense in an animal with sudden and massive input of protein.

The allatostatins (AS) and allatropins (AT) are multifunctional neuropeptides and AS occur in virtually all insects investigated (Gäde et al., 1997). Depending on the species, these neurohormones stimulate and/or inhibit simultaneously juvenile

hormone synthesis, gut myoactivity, and the release of digestive enzymes (Gäde et al., 1997; Lorenz, 2001; Aguilar et al., 2003). In the larvae of the coconut pest *Opisina arenosella,* various FMRF amides, leucomyosupressins, stimulate the in vitro release of amylase and protease (Harshini et al., 2002b). Spofr-AS A5 and AS A6 belong to the allatostatin type-A family. AT and AS bind to receptors on the hemolymph side of the enzyme-producing cells (zymogen cells) and induce either an inhibition or stimulation of the release on the lumen end, for example the stimulation of amylase release in response to AS in the cockroach *D. punctata* (Fúse et al., 1999). Based on gene sequence studies, Manse-AT and Spofr-AS A5 and AS A6 most likely occur in *S. frugiperda* (Abdel-latief et al., 2004). Spofr-AS A5 moderately inhibits amylase and trypsin release in *S. frugiperda*, but AS A6 does not. Both peptides belong to the FGLa family and are about the same size, but the amino acids sequences are quite different. The allatotropin Manse-AT has quite the opposite effect, stimulating the release of amylase and trypsin.

Why do peptides and amino acids have no effect on the release of trypsin (current study) though AS and AT clearly inhibit or stimulate trypsin release, respectively? A possible explanation is that peptide receptors on the lumen side of the neurohormonal cells are lacking in this species, though the AT and AS receptors on the hemolymph side of the zymogen cells may be present. Blood-sucking insects, on the other hand, have the peptide receptors on the lumen side of the neuroendocrine cells, and, therefore, dietary peptides induce trypsin release (Blakemore et al., 1995).

Several myosuppressins of the FLRFamide family also stimulate amylase release, for example in *L. migratoria* (Hill and Orchard, 2005), the beetle *Rhynchophorus ferrugineus* (Nachman et al., 1997), and in the lepidopteran *Opisina arenosella* (Harshini et al., 2002b). Leucokinines, on the other hand, inhibit amylase and general protease release while stimulating midgut motility in *Opisina arenosella* (Harshini et al., 2002a). AT stimulates foregut muscles in several lepidopterans (Duve et al., 1999; Matthews et al., 2007), and an FGLamide (AS type-A) and a WX_6W allatosttain (AS type-B) inhibit gut myocontraction in *G. bimaculatus* (Lorenz, 2001) and *M. sexta* (Blackburn et al., 1995). Interestingly, in almost all other Lepidoptera larvae an in vitro contraction of the foregut is seen. However, in *S. littoralis* (Matthews et al., 2008) and *S. frugiperda* (current study), foregut motility is not observed. Therefore, in the current study the ilium was employed to assay myoactivity. In view of the fact that some caterpillars show both stimulatory and inhibitory responses (above), it is not surprising that in the current study an opposing response to the tested ASTs and AT occurs. Manse-AT is myostimulatory and Spofr-AS A5 myoinhibitory. A similar interaction also is reported in another lepidopteran, *Lacanobia oleracea* (Matthews et al., 2007). In summary, Spofr-AT stimulates digestive enzyme release and myoactivity in *S. frugiperda* and Spofr-AS A5 inhibits both.

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Article

EXOGENOUS AND ENDOGENOUS PROTEASE INHIBITORS IN THE GUT OF THE FALL ARMYWORM LARVAE, *Spodoptera frugiperda*

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A dose-dependent inhibition of endogenous trypsin and aminopeptidase occurs in the lumen of Spodoptera frugiperda after feeding L6 larvae exogenous inhibitors soybean trypsin inhibitor (SBTI), tosyl-L-lysine chloromethyl ketone-HCl (TLCK), or bestatin, respectively, for 3 days. TLCK inhibits trypsin in tissue extracts and in secretions more strongly than SBTI. The aminopeptidase released into the lumen (containing the peritrophic membrane) is strongly inhibited by bestatin, but the membrane-bound enzyme is not. A bound enzyme may be more resistant to an inhibitor than unbound. A cross-class elevation of aminopeptidase activity occurs in response to ingested trypsin inhibitor, but there was no cross-class effect of aminopeptidase inhibitor (bestatin) on trypsin activity. An endogenous trypsin and aminopeptidase inhibitor is present in the lumen and ventricular cells. The strength of the endogenous trypsin inhibition seems to be in the same range as that resulting from ingestion of the exogenous inhibitor SBTI. In some insect species, considerable trypsin secretion occurs in unfed as well as in fed animals, and endogenous protease inhibitors might function to protect the ventricular epithelium by inactivation of trypsin when less food is available. © *2010 Wiley Periodicals, Inc.*

Keywords: enzyme secretion; lumen contents; epithelium; dose response; trypsin inhibitors; aminopeptidase inhibitors

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INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) is an important pest of corn, sorghum, cotton, and alfalfa in the tropical regions of the Western hemisphere. It has proved to be an ideal model insect for the study of the basic functions of the digestive system in defoliating caterpillars. The distribution of digestive enzymes, the role of the peritrophic membrane (PM), and the counter-current flow of enzymes in *S. frugiperda* is well documented (Ferreira et al., 1994a,b; Bolognesi et al., 2001). The release of digestive enzymes in the larvae of the armyworm is regulated by the release of neuropeptides in response to the presence of specific nutrients in the lumen (Lwalaba et al., 2009).

Exogenous protease inhibitors in insects are produced by many plants as a defence against feeding (Fan and Wu, 2005), and in general they have their effect in the crop or midgut where they inhibit the proteases present in the lumen (Johnston et al., 1993; Telang et al., 2005; Duncan et al., 2006; Brioschi et al., 2007) as well as the secretion of proteases by the epithelium (Lwalaba et al., 2009). These exogenous protease inhibitors are Kunitz or Kazal type serine protease inhibitors.

Endogenous serine protease inhibitors are widely found in all tissues of all animals. The serpins, form a very large class of proteases, are mostly intracellular, and undergo a unique change in shape when they inhibit target proteases (Huntington et al., 2000). They regulate such processes as coagulation, inflammation, and immunity. Thus far, all known insect serpins are involved in insect immunity (Abraham et al., 2005). However, some insect inhibitors involved with immune responses (coagulation, prophenol oxidase activation, etc.) belong to the classical Kunitz and Kazal type inhibitors (Kanost, 1999), that is, they bind to and block access to the protease active site (Kunitz, 1948). These endogenous inhibitors are produced by blood cells, silk glands, or ovaries (Nirmala et al., 2001; Simonet et al., 2002; Brillard-Bourdet et al., 2006). Protease inhibitors of the Kazal type are found in the salivary gland and saliva of *Nauphoeta cinerea*, which are particularly effective against the bacterial protease subtilisin. This suggests a defensive mechanism against the masses of bacteria typically found in the cockroach crop, some of which are probably pathogenic (Taranushenko et al., 1909). Inhibitory peptides in the midgut lumen are less well studied, though trypsin and subtilisin inhibitors are described from whole midgut preparations of the cockroach *N. cinerea* (Elpidina et al., 2001), and in five additional cockroach species (Vinokurov et al., 2007).

The first aim of the current study is to document the exogenous inhibition of protease activity in the lumen and epithelial cells of the midgut and further document the inhibition of protease secretion in L6 larvae of *S. frugiperda*. The cross-class inhibition of exogenous aminopeptidase by trypsin inhibitors (SBTI) and vice versa is likewise described. The major aim, however, is to determine if endogenous trypsin and aminopeptidase inhibitors are produced by the midgut cells and released into the lumen. Finally, the relative strength of inhibition of exogenous inhibitors is compared to the endogenous inhibitors.

MATERIALS AND METHODS

Rearing Methods, Diets, and Inhibitors

Pupae and eggs of *S. frugiperda* and an artificial diet (based on bean meal) were provided by Bayer Crop Science AG (Monheim, Germany). Larvae were reared at

27°C and approximately 70% relative humidity under a L16:D8 photoperiod as previously described (Oeh et al., 2000). To prevent cannibalism, L4 larvae were maintained individually in separate compartments of assortment boxes with 40 compartments (49 × 32 × 36 mm per compartment). In order to test the effect of diet and feeding on enzyme and inhibitor activity, newly moulted L6 were provided a cellulose diet (30 g cellulose powder +3 g agar in 250 ml water) instead of the control diet, and others received no food at all. The ventriculus of L6 day-3 larvae was used for animals fed the control diet and the non-nutritive cellulose diet. Under these conditions, the gut of fed larvae degenerated early on day 4 and the larvae entered the prepupal stage. The digestive tract of unfed larvae started to regress earlier into the prepupal condition on day 3, therefore the ventriculus of 2-day-old unfed larvae was used. Soybean trypsin inhibitor (SBTI), the specific trypsin inhibitor (synthetic) tosyl-L-lysine chloromethyl ketone-HCl (TLCK), and the aminopeptidase inhibitor bestatin (from the bacterium *Streptomyces olivoreticuli*) were dissolved in water and added to a fresh 300-mg cube of the Bayer artificial diet or cellulose diet on each of the 3 days of the L6 larvae (in vivo). The concentration of SBTI tested was 0.1 to 0.4%, of TLCK was 0.025 to 0.15%, and of bestatin 0.03 to 0.10%. TLCK inhibited feeding at concentrations >0.15%, and were therefore not tested. The diet was generally completely or almost totally consumed by L6 larvae in 24 h. In vitro inhibition was tested by adding the inhibitor to 200 µl of an extract of the ventriculus.

Ventriculus Dissection: Source of Endogenous Enzymes

Larvae of *S. frugiperda* were anesthetized on ice, slit open, and the entire ventriculus removed. A crop and caeca are lacking, and functionally the entire midgut consists of the ventriculus. The standing enzyme activity in the tissue homogenate and in the lumen content, which included the exo- and endoperitrophic space plus the peritrophic membrane (PM), was measured. The total lumen content of one ventriculus was placed in 300 µl *Spodoptera* Ringer (per Liter 0.7 g NaCl, 2.0 g KCl, 0.25 g $NaHCO_3$.2 H_2O, 1.3 g $CaCl_2$.2 H_2O, 4.4 g $MgCl_2$.7 H_2O, 1.0 g Hepes at pH 7.0). Two ventriculi were thoroughly rinsed in Ringer and placed in 300 µl Ringer, lightly sonicated (Bronson Sonifier 250, 2–3 sec at the lowest setting) and centrifuged. Two freshly dissected ventriculi were slit open and rinsed with low-glucose Ringer (LGR) (10 mg glucose/100 ml Ringer), a concentration adapted from Fusé et al. (1999) for incubation of gut tissues of the cockroach *Diploptera punctata*, and placed in 300 µl fresh LGR, and incubated for 30 min at 30°C without shaking. The incubated tissue was discarded, and the remaining incubation medium was used to measure enzyme release (secretion). Thus, there were three sources of enzymes: lumen content, tissue homogenate, and the secretion in the incubation medium.

Endogenous Enzyme Assays

For the determination of protease activity 100 µl of the lumen contents, tissue homogenate, or the incubation medium was used for the endogenous trypsin and aminopeptidase assay. The trypsin and aminopeptidase activities were linear from 0 to 5 min at 25°C at a pH of 8.0 (data not shown). For both assays, the release of NA (nitroaniline) (dAbs/min) from the synthetic substrates α-N-benzoyl-DL-arginine-p-NA (BApNA) and L-leucine-p-NA (LpNA), respectively, was measured at 405 nm. A 100-µl sample was added to 700 µl 1 mM BApNA (4.3 mg/10 ml Tris buffer) or to 700 µl 1 mM LpNA (2.87 mg/10 ml). One unit of activity was the amount of enzyme required to split

1 µmol of the substrate, which was calculated from extinction coefficient, $\Delta Abs \times 10^6/9{,}900$.

Determination of Endogenous Trypsin Inhibitors

All enzymes, except those from hot-spring bacteria, are inactivated by temperatures over 80°C. It was already observed many years ago (Kunitz, 1948) that SBTI is denatured at 60°C and higher, but upon cooling the native form is restored. However, to be quite sure, the exogenous enzymes (bovine trypsin, and aminopeptidase from *Aeromonas proteolytica*) and the inhibitors (SBTI and bestatin) were exposed to 90°C for 10 min. The enzymes were indeed irreversibly inactivated (>98% loss of activity) and the exogenous inhibitors were <10% inactivated.

In order to demonstrate the presence of endogenous inhibitory activity, two rinsed ventriculi were incubated in 300 µl LGR for 30 min at 30°C, two rinsed ventriculi were sonicated with a Bronson Sonifier 250 in 300 µl LGR, and the content of one ventriculus was brought up to 300 µl in LGR. From each of these samples, 150 µl was heated to 90°C for 10 min, and the other 150 µl was not. Both were again centrifuged. The control was LGR. To 100 µl of each of these samples (3 heated, 3 not heated, +control), 100 µl of the exogenous enzyme bovine trypsin (10 µg/100 µl) or aminopeptidase (0.25 U/100 µl) was added. The heated samples contain no enzymes, but should contain active endogenous inhibitors. Mild shaking at 30°C for 5 min allowed time for the endogenous inhibitor to bind to the exogenous enzyme. From this 200-µl mix, 100 µl was added to 700 µl BApNA or LpNA and the exogenous trypsin or aminopeptidase activity was measured (U/ventriculus/min). The control (no inhibitor) was set to 100%, and the change in activity (bovine trypsin or bacterial aminopeptidase) was converted to % change. The differences between heated and unheated lumen contents, tissue extracts, and tissue secretions, respectively, were determined for larvae fed the standard diet, a non-nutritive cellulose diet, and for larvae not fed at all. Unless otherwise stated, the exogenous enzymes and inhibitors were obtained from Sigma (Taufkirchen, Germany).

Visualisation of Endogenous Trypsin Activity by SDS-PAGE

For the separation by analytical SDS-PAGE samples were applied onto 15% polyacrylamide gels (10 × 10 cm; 3.0 mm thick) with 0.1% SDS at a pH of 8.8. Ten microliters of heated lumen extracts (enzymes inactivated) were added to 10 µl of non-reducing (no DTT) electrophoresis sample buffer (1 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 2% bromophenol blue) just before loading onto the gel. Electrophoresis was carried out at room temperature at 100 V constant voltage.

Gelatine-SDS-PA gels were cast with 12% acrylamide, and 0.1% gelatine as described by Hanspal et al. (1983). The stacking gel contained 5% acrylamide and no substrate. Samples (20–30 µl trypsin, SBTI and lumen contents) were dissolved in aqueous solution of non-reducing (no DDT) electrophoresis sample buffer (the same as used for analytical SDS-PAGE) and applied to the gel. Gelatine gels were run at constant 100 V, 35 mA for 4 hr. After migration, the gels were washed in a 2.5% Triton X-100 in 100 mM Tris-HCl buffer pH 8.5 for 45 min and 3 times in distilled water (10 min each time) at room temperature. Then the gels were incubated in 100 mM Tris-HCl pH 8.5 containing 0.5 mg trypsin/ml at 37°C overnight. After incubation the gels were again washed and stained with Page Blue Coomassie R-250 (Fermentas).

The bound gelatine-inhibitors were not digested by the trypsin and appear as thick blue bands on a clear background.

Statistical Analysis

The Win Stat program for Microsoft Excel (Redmond, WA) was used to evaluate the data. The differences in % change in enzyme activities were tested with the MannWhitney U-test. Statistical significance is shown in Figures 1 and 2 and Tables 1–3 ($^{*}P<0.05$, $^{**}P<0.01$).

RESULTS

Exogenous Inhibition of Ventricular Enzymes

The standing trypsin activity in the lumen of diet fed L6 larvae was fairly high (4.0 U/min), compared to the much lower activity in the epithelial cells (0.2 U/min), or that released from the cells in 30 min of incubation (0.2 U/min). The aminopeptidase standing activity in the lumen, on the other hand, was much lower (2.5 U/min) compared to the cells (20 U/min). This is in accordance with the known distribution of these two enzymes in insects, where trypsin is quickly released into the lumen and aminopeptidases remain mostly bound to the epithelial membranes (Ferreira et al., 1994a,b). The control activity of the secretion was 0.67 U/min.

A strong inhibition of trypsin activity in the ventricular lumen by the exogenous inhibitors SBTI and TLCK, and aminopeptidase by bestatin was apparent in L6 larvae fed the inhibitors over 3 days (Fig. 1). A distinct in vivo dose-response inhibition for all three inhibitors was apparent in the lumen. In general, TLCK was a stronger inhibitor than SBTI. Both SBTI and TLCK inhibited trypsin activity in the tissues. SBTI inhibited trypsin release only at a high concentration (0.4%), while TLCK inhibited secretion at concentrations as low as 0.05%. Bestatin strongly inhibited aminopeptidase in the lumen but had no effect on the secretion or tissue-bound activity of aminopeptidase (Fig. 1C).

These same inhibitors when added directly to the cellular extracts (in vitro) strongly inhibited all enzymes. Compared to control activity (set to 100%), the in vitro trypsin activity was reduced to $24\pm7\%$ by SBTI, to $9\pm3\%$ by TLCK, and aminopeptidase activity was reduced to $15\pm2\%$ by bestatin. Thus, it was clear that the inhibitors used specifically inhibited the enzymes released by the caterpillar and not just the exogenous enzymes used for the enzyme assays.

Cross-Class Reactions of Exogenous Trypsin and Aminopeptidase Inhibitors

SBTI fed to L6 larvae for 3 days strongly inhibited trypsin, and it also induced a moderate elevation ($P<0.05$) of aminopeptidase activity in the lumen. The more potent trypsin inhibitor TLCK more strongly elevated ($P<0.01$) aminopeptidase activity in the lumen and in addition also in the tissue extracts (Table 1). Thus, aminopeptidase showed an in vivo cross-reaction to both protease inhibitors (SBTI and TLCK). The aminopeptidase inhibitor bestatin, however, had no in vivo cross-class effect on the trypsin activity.

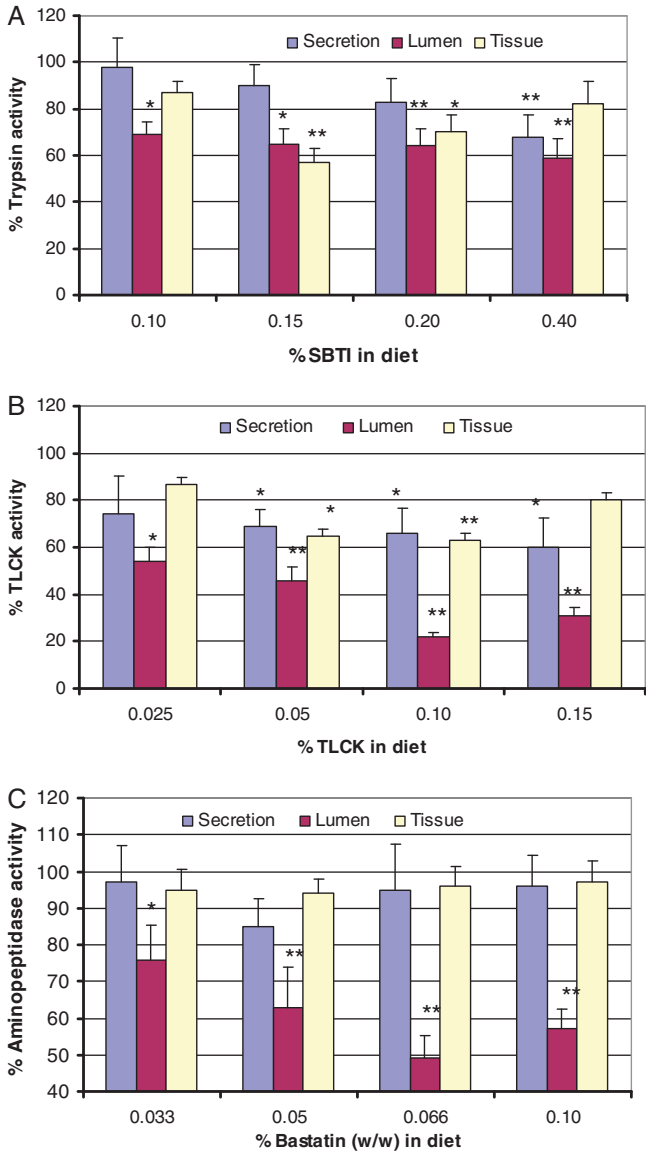

Figure 1. Effects of exogenous inhibitor concentration on the enzyme activity in the tissue secretions, tissue homogenates, and lumen content of the ventricle of L6 (day 3) *S. frugiperda* larvae fed for 3 days on the standard diet containing SBTI, TLCK, or bestatin. **A:** Soybean trypsin inhibitor (SBTI), a serine protease inhibitor. **B:** Tosyl-L-lysine chloromethyl ketone-HCl (TLCK), a trypsin-specific inhibitor. **C:** Bestatin, an aminopeptidase inhibitor. The control trypsin activity in the lumen of diet-fed L6 larvae (no inhibitor) activity was 4.0 ± 0.5 U/ventriculus/min, in the cells 0.2 U/vent/min, and in the secretion (30 min) 0.2 U/min. The aminopeptidase activity in the lumen was 2.5 U/ventriculus/min, in the cells 20.0 U/ventriculus/min, and in the secretions 0.67 U/ventriculus/min. The response to the inhibitors was converted to % change in activity compared to control activity (set to 100%). Standard error of mean is given as vertical bars. *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$.

Endogenous Inhibition of Ventricular Enzymes

The endogenous inhibitors in the heated tissue extracts in diet-fed L6 larvae showed less trypsin activity (78%), and much less aminopeptidase activity (47%) compared to

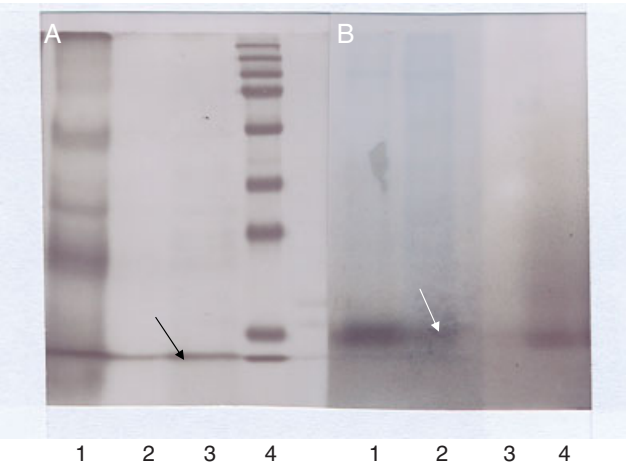

Figure 2. **A:** Analytical SDS-PAGE. Samples were applied onto 15% polyacrylamide gels with 0.1% SDS at a pH of 8.8. Extracts (10 µl) were added to 10 µl of non-reducing (no DTT) electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 2% bromophenol blue) without heating just before loading onto the gel. Lane 1: Lumen contents (heated) of diet-fed larvae; lane 2: unconcentrated lumen contents of cellulose-fed larvae, lane 3: 3 × concentrated lumen contents (heated) of cellulose-fed larvae; lane 4: Molecular weight standards (11,17,18.36, 55, 72, 95, 130, 250 kDa). Inhibitor marked by black arrow. **B:** Gelatin-SDS-PAGE. Gels were cast with 12% acrylamide and 0.1% gelatine. The stacking gel contained 5% acrylamid and no substrate. Samples (trypsin, SBTI, and lumen contents; 20–30 µl) were dissolved in electrophoresis sample buffer (the same as used for analytical SDS-PAGE) and applied to the gel without heating. After migration, the gels were washed in a 2.5% Triton X-100 in 100 mM Tris-HCl buffer, pH 8.5, for 45 min and 3 times in distilled water. Then the gels were incubated in 100 mM Tris-HCl, pH 8.5, containing 0.5 mg trypsin/ml at 37°C overnight. After incubation, the gels were again washed and stained with Page Blue Coomassie R-250. The bound gelatine-inhibitors were not digested by the trypsin and appear as thick blue bands on a clear background (white arrow). Lane 1: lumen contents of diet-fed larvae; lane 2: lumen contents of cellulose fed larvae; lane 3: 5 µg bovine trypsin; lane 4: 25 µg SBTI. Arrow indicates endogenous trypsin inhibitor.

Table 1. Cross-Class Reactions of Endoprotease Inhibitors and Exopreotease Inhibitors*

Dose of inhibitor	*Enzyme*	*Lumen*	*±SEM*	*Tissue*	*±SEM*	*Secretion*	*±SEM*
0.20% Bestatin	Adase	57**	5.5	97	6	96	6
0.10% SBTI	Adase	142*	13	113	3	136	13
0.10% TLCK	Adase	188*	17	120*	3	115	10
0.10% SBTI	Trypsin	69**	5.2	67**	5	98	12
	Trypsin	96	7.6	99	7	101	7

*SBTI and TLCK are endoprotease (trypsin) inhibitors and bestatin is an aminopeptidase (Adase) inhibitor. The dose is givien as % inhibitor in the diet (w/w). L6 larvae were fed the inhibitor for 3 days, and enzyme activity was tested on day 3. The activity of the Adase and trypsin are given as % change in untreated controls set to 100%. The control activities (U/min) are as given in Figure 1. $N = 10$, $^{*}P < 0.05$ and $^{**}P < 0.01$ Student's *t*-test.

controls (100%) (Tables 2 and 3), which demonstrated the presence of endogenous inhibitors in the ventricular cells. The unheated tissue extracts resulted in more trypsin (143%), and very much more aminopeptidase activity (332%), a result of the combination of active endogenous enzymes plus the added exogenous enzymes. Since the tissues were thoroughly rinsed, exogenous inhibitors associated with the food were removed, and only endogenous inhibitors were present in the heated tissue extract.

Table 2. Inhibitory Effects of Ventricular Incubation Medium, Tissue Homogenates, and Lumen Contents on the In Vitro Bovine Trypsin Activity in Control-Fed (3d), Non-Nutritive-Fed (3d), and Unfed (2d) L6 Larva of S. frugiperda[†]

	Diet fed		*Cellulose fed*		*Unfed*	
Sample (100 µl)	*Rel Act*	*±SEM*	*Rel Act*	*±SEM*	*Rel Act*	*±SEM*
Control (9.3 U/min)	100	0	100	0	100	0
Heated secretion	109	2	93	2	95	3
Unheated secretion	124	5	114	3	113	3
Heated tissue homogenate (cells)	78**	3	89*	2	80*	3
Unheated tissue homogenate	143	6	104	3	133	3
Heated lumen contents	65**	4	81*	3	78*	4
Unheated lumen contents	426	9	152	6	192	6

[†]Relative activity (Rel Act) = % activity compared to controls set to 100%. The higher enzyme activity in the unheated tissue extracts and lumen results from insufficient inhibitor to inactivate total enzymes (both endogenous +exogenous). Controls contained no enzyme or inhibitor. Heated samples contained inactive endogenous enzyme plus active endogenous inhibitors. Unheated samples contained active endogenous enzymes plus active endogenous inhibitors. The exogenous enzyme bovine trypsin was added to control, heated, and unheated samples and 10 min incubation was allowed for the endogenous inhibitor to bind to the trypsin. BApNA was added to a subsample and the enzyme activity assayed as described in Materials and Methods. One unit of enzyme activity is the amount of enzyme necessary to split 1 µmol of substrate.
*$P < 0.05$, moderate inhibition; **$P < 0.01$, strong inhibition, (t-test) $N = 10$.

Table 3. Inhibitory Effects of Ventricular Incubation Medium, Tissue Homogenates, and Lumen Contents on the In Vitro Aminopeptidase Activity in Control-Fed (3d), Non-Nutritive-Fed (3d), and Unfed (2d) L6 Larvae of S. frugiperda[†]

	Diet fed		*Cellulose fed*		*Unfed*	
Sample (100 µl)	*Rel Act*	*±SEM*	*Rel Act*	*±SEM*	*Rel Act*	*±SEM*
Control (34.5 U/min)	100	0	100	0	100	0
Heated secretion	77	7	85	2	97	3
Unheated secretion	100	3	104	3	102	3
Heated tissue homogenate (cells)	47**	2	75*	5	94*	3
Unheated tissue homogenate	332	12	312	10	169	10
Heated lumen contents	79*	3	94	3	98	6
Unheated lumen contents	94	3	133	4	92	6

[†]The enzyme bacterial aminopeptidase was added to control, heated, and unheated samples and 10 min were allowed for the endogenous inhibitor to bind to the exogenous enzyme. Other abbreviations as in Table 2. LpNA was added to a subsample and the enzyme activity assayed as described in Materials and Methods.
*$P < 0.05$, moderate inhibition; **$P < 0.01$, strong inhibition, (t-test) $N = 10$.

The inhibitors in the heated lumen contents resulted in much less trypsin (65%) and aminopeptidase (79%) activity (Tables 2 and 3), clearly an indication of endogenous inhibitors. The unheated lumen contents showed very high trypsin activity (426%), because both endogenous and exogenous trypsins were present. A similar endogenous trypsin inhibition was likewise observed in cellulose-fed or unfed L6 larvae. It should be noted that there could not be any exogenous inhibitors in the lumen of cellulose-fed or unfed larvae.

The situation with endogenous aminopeptidase inhibition is different from that of trypsin inhibition because most aminopeptidase is not released into the lumen, as is

trypsin, but rather remains bound to the cell membranes (Ferreira et al., 1994a). The membrane-bound aminopeptidases of heated tissue extracts were inactivated, and the endogenous aminopeptidase inhibitors present led to greatly reduced ($P < 0.01$) activity in the exogenous aminopeptidase (Table 3). The PM is part of the lumen contents, and it contains some aminopeptidase (Ferreira et al., 1994a), and a moderate amount ($P < 0.05$) of endogenous aminopeptidase inhibition was found in heated lumen contents. There was a moderate inhibition of aminopeptidase activity in the tissues of cellulose-fed larvae, but no inhibition in starved larvae.

The analytical SDS-PAGE indicated a peptide present in the heated lumen contents of diet-fed and cellulose-fed L6 larvae, which has a molecular weight in the range of 13–15 kDa (dark arrow, Fig. 2A). All enzymes were inactivated by heating 10 min at 90°C and do not appear in the gel. The zymogram (gelatine-SDS-PAGE) of the endogenous trypsin inhibitor in the heated lumen sample indicated that the inhibitor bound to the gelatine (white arrow, Fig. 2B) and prevented its digestion by the bovine trypsin incubation (see Materials and Methods).

Comparison of Exogenous and Endogenous Inhibitors

It is difficult to compare the effectiveness of exo- and endogenous protease inhibitors. The inhibition of the exogenous inhibitors (ingested plant material) was measured as the decrease in the endogenous trypsin and aminopeptidase activity in the ventriculus. The inhibition of the endogenous inhibitors (released from ventricular cells) was measured as the decrease in digestion of exogenous trypsin (bovine) and aminopeptidase (bacterial) by lumen contents and tissue homogenates. However, an approximate comparison can be made. Endogenous luminal trypsin was inhibited to 65% (Fig. 1, with 0.2% SBTI, and the activity was reduced from 4.0 U/min to 2.6 U/min). Exogenous trypsin was inhibited in the lumen also to 65% (Table 2) and the activity was reduced from 9.3 U/min to 6.0 U/min.

DISCUSSION

A wide variety of enzyme inhibitors occur in plants (see review by Fan and Wu, 2005), and an inhibition of digestive enzymes in insects can result from ingestion of plants containing one or more of these inhibitors. Protease inhibition by exogenous inhibitors (plant origin) occurs in *S. frugiperda* (Alfonso et al., 1997; Brioschi et al., 2007, current study) as well in many other lepidopteran larvae (Christeller et al., 1992; Johnston et al., 1993; Telang et al., 2005). In the current study, inhibition of digestive enzyme secretion by the gut epithelium in addition to inhibition of the enzymes already present in the lumen was demonstrated. This is the first report of the direct inhibition of enzyme release.

With regards to exogenous inhibitors in the lumen, the more specific trypsin inhibitor TLCK (a synthetic product) appears to be a stronger inhibitor of trypsin than the general serine protease inhibitor SBTI, because about three times more SBTI is required to cause a similar inhibiton of trypsin as TLCK. Both SBTI and TLCK inhibited activity of trypsin in cell homogenates and in the secretions. Inhibition of an enzyme secretion implies either a direct effect (entry into the cell) or indirect effect (docking on receptors), which then has an effect on the cell production (synthesis) and/or release of the enzyme. This is not surprising, because there is already considerable evidence for the effect of inhibitors on increased or altered trypsin variants in the gut

epithelial cells of *S. frugiperda* and *Heliothis virescens* in response to acute feeding of SBTI (Lara et al., 2000; Brito et al., 2001; Brioschi et al., 2007).

Aminopeptidase is mostly bound to cell membranes, and only about 17% occurs in the PM of *S. frugiperda* (Ferreira et al., 1994b), which is included in the lumen content in the current study. The aminopeptidase activity of the epithelium (membrane bound) was not inhibited by the exogenous inhibitor bestatin, but the small amount (17%) associated with the PM in the lumen was strongly inhibited. It is possible that being bound to the cell membrane somehow protects the aminopeptidase.

Cross-class inhibition of plant protease inhibitors, as reported in the current study, occurs fairly frequently in insects. This indicates an altered synthesis of one enzyme in response to a reduction of a digestion product of a different enzyme. In *Teleogryllus commodus*, SBTI also increases aminopeptidase activity (Burgess et al., 1994), as does a barley trypsin inhibitor in *S. exigua* (Lara et al., 2000). The barley inhibitor in *S. frugiperda* inhibits both trypsin, but has no effect on chymotrypsin, elastase, or aminopeptidase (Alfonso et al., 1997). A molecular basis of cross-class effects is seen in *Helicoverpa armigera*, where specific inhibitors increase chymotrypsin mRNA but trypsin mRNA levels decrease (Gatehouse et al., 1997), or in *Trichoplusia* ni, where a procarboxypeptidase is activated by trypsin (Wang et al., 2004).

The many endogenous proteases associated with various insect tissues (blood, ovaries, and fat body) are almost all involved with immunity or their function is unknown (Hamdaoui et al., 1998; Kanost, 1999; Simonet et al., 2002). The first demonstration of endogenous protease inhibitors specifically in the gut was described in tissue extracts and lumen contents of the midgut of the cockroach *Leucophaea maderae* (Engelmann and Geraerts, 1980). They used boiled extracts to inactivate endogenous enzymes, as in the current study, and they found up to 80% protease inhibition in the posterior half of the midgut. Recently, endogenous protease inhibitors have been described in the digestive system of cockroaches (Taranushenko et al., 1909; Elpidina et al., 2001) using whole gut extracts (epithelium plus lumen contents). Inhibition of trypsin and aminopeptidase occurs in the tissue extracts and in the lumen of *S. frugiperda* (current study), which is the first report of endogenous digestive enzyme inhibitors in a lepidopteran species.

Several functions of the endogenous protease inhibitors in the digestive tract of cockroaches have been suggested. Elpidina et al. (2001) suggests that the endogenous protease inhibitors could protect amylases in the anterior midgut of *N. cinerea*, which might be true in some insects. However, divalent ions, especially Ca, seem to confer a general resistance of α-amylase to proteolytic degradation in vertebrates (Stein and Fischer, 1958), and this probably holds true in *S. frugiperda*. Other authors suggest that endogenous protease inhibitors might be important as protection against fungi and bacteria that employ subtilisins to attack insect tissues (Taranushenko et al., 2009; Vinokurov et al., 2007). This seems to be especially appropriate in cockroaches, where the salivary inhibitors could protect the insect against the massive amounts of bacteria that are stored over long periods in the crop (Taranushenko et al., 2009).

Another possible function of endogenous protease inhibitors in some insects, which has not been considered, might be protection of the epithelium when dietary protein is scarce or lacking. In vertebrates, trypsinogen is stored in zymogen cells of the pancreas and secreted in response to food and the release of cholycystokinin. When protein is lacking, trypsinogen is not secreted. In *S. frugiperda* and in *G. bimaculatus,* very little trypsin is stored in the epithelial cells, but instead it is immediately and continuously released. In unfed individuals, the basal trypsin activity

in the midgut is 1/3 to 1/2 of that present in fed animals (Woodring et al., 2009, current study). The constant release of active trypsin may perhaps be normal in continuous feeding insects (such as crickets and caterpillars), but this could pose a threat to the epithelium when food intake is restricted. An endogenous trypsin inhibitor would inactivate the trypsin when too little protein is present in the ventriculus.

In conclusion, the digestive enzymes of *S. frugiperda* are susceptible to both exogenous and endogenous inhibitors. Acute feeding of exogenous inhibitors (SBTI and TLCK) to L6 larvae reduces trypsin activity in the lumen, in the cells, and in the cell secretions. The exogenous aminopeptidase inhibitor bestatin inhibits aminopeptidase released into the lumen, but has no effect on aminopeptidase bound to the epithelial membranes. This would protect most of the aminopeptidase from inhibitors. A cross-class stimulation of aminopeptidase activity in response to exogenous trypsin inhibitor is observed but not the reverse. Endogenous trypsin and aminopeptidase are present in the lumen and tissue extracts of the ventriculus. Since active trypsin is secreted also in unfed larvae, it is suggested that the endogenous protease inhibitors might function to protect the exposed epithelium when food is not present in the lumen.

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Summary

This thesis investigates control of the release of digestive enzymes in the cricket *Gryllus bimaculatus* and the fall armyworm, *Spodoptera frugiperda*.

1. Control of enzyme release in the cricket *G. bimaculatus*. Using flat-sheet preparations of the caecum, digestive enzyme release was investigated. More trypsin, aminopeptidase and amylase are secreted in the caecum of fed crickets than in unfed crickets, but basal levels of certain enzymes are released continuously even in unfed animals. A variable ratio of nutrients in ingested food leads to a different ratio of digestive enzyme release, but a high nutrient component in the food does not necessarily induce a high digestive enzyme release for that component. Maltose and glucose elevate amylase release from the tissues into the incubation medium, but starch does not. Bovine serum albumin (BSA), peptone and a mixture of amino acids have almost no effect on the release of aminopeptidase, and only low concentrations of peptone increase trypsin release. In crickets, the continuous release of proteases is sufficient to meet the needs for growth, and only moderate stimulation of trypsin results from feeding. Carbohydrates are used for energy, and the release of amylase is adjusted to the amount of food ingested. The neuropeptide allatostatin Grybi-AS 5 elevates the release of amylase in fed females, but not of trypsin or aminopeptidase, however, both amylase and trypsin release are stimulated by AS 5 in unfed crickets. Fed crickets have sufficient trypsin to obtain needed amino acids, but unfed do not, therefore the AS stimulation of trypsin release in unfed crickets makes sense.

2. Control of enzyme release in the larvae of *S. frugiperda*. A flat-sheet preparation of the ventriculus was used to test the release of amylase, trypsin and aminopeptidase in response to specific nutrients in the food and to specific neuropeptides. The epithelial secretion rate of amylase and trypsin was about 20% of the amount of enzyme present in the ventricular lumen, which, considering the efficient counter-current recycling of enzymes, suggests that the secretion rate is adequate to replace egested enzymes. Dietary carbohydrates are used for energy, and larvae adjust amylase activity

to carbohydrate ingestion. Amylase activity is 5-times higher in fed compared to unfed larvae, and sugars in the incubation medium induces more than a 300 % increase in amylase release. Plants contain a low level of protein, but larvae need proteins for growth, thus the larvae can not afford to lose proteins by egestion. Therefore, trypsin activity remains high even in unfed larvae. As a result, proteins and amino acids have little or no effect on trypsin or aminopeptidase release in incubated tissues. The control of enzymes release in response to food is most likely mediated through neurohormones. Indeed, an allatostatin (Spofr-AS A5) inhibits amylase and trypsin release, and allatotropin (Manse- AT) stimulates amylase and trypsin release. Spofr-AS A5 also inhibits ileum myoactivity and Manse- AT stimulates myoactivity.

3. Inhibition of enzyme release in the larvae of *S. frugiperda*. Exogenous inhibitors are produced by plants, and are ingested by the insect. Endogenous inhibitors are produced by the gut epithelial cells themselves. A dose-dependent inhibition of endogenous enzymes occur in the lumen after feeding L6 larvae with the exogenous serine protease inhibitor from soybean (SBTI), the specific trypsin inhibitor TLCK, an aminopeptidase inhibitor (bestatin), and an amylase inhibitor from wheat. Inhibition in tissue extracts is seen only with higher doses of SBTI and TLCK. Inhibition of enzyme release into the incubation medium is apparent only with very high doses of SBTI. Inhibition in the tissues and inhibition of release indicate a direct cellular response to an inhibitor present in the lumen. The elevation of aminopeptidase activity in response to ingested trypsin inhibitors indicates a cellular synthesis in response to the product of a digestive enzyme. The enzymes investigated are irreversibly inactivated by 10 min at 90°C, but the corresponding inhibitors are not, therefore endogenous inhibitors could be identified. Endogenous inhibitors are present in the ventricular cells and in the lumen. We suggest that the endogenous protease inhibitors may protect the epithelium by inactivation of the trypsin in underfed larvae. This is the first explanation of how insects are able to secrete an active trypsin.

Zusammenfassung

Diese Dissertation untersucht die Steuerung der Freisetzung der Verdauungsenzyme bei der Mittelmeerfeldgrille *Gryllus bimaculatus* und dem Maisschädling *Spodoptera frugiperda.*

1. Steuerung der Enzymfreisetzung bei der Mittelmeerfeldgrille *G. bimaculatus*. Unter Verwendung von flachgeschnitten Gewebepräparaten des Darmcaecums wurde die Freisetzung der Verdauungsenzyme untersucht. Mehr Trypsin, Aminopeptidase und Amylase werden im Caecum von gefütterten Grillen freigesetzt als bei ungefütterten Grillen, aber eine gewisse Menge an Enzymen wird kontinuierlich auch bei ungefütterten Tieren freigesetzt. Verschiedene Nährstoffe im aufgenommenen Futter führen zu einem weiteren Anteil an Verdauungsenzymfreisetzung, obwohl ein hoher Nährstoffbestand in der Nahrung nicht unbedingt eine hohe Freisetzung von Verdauungsenzymen für diesen Bestandteil hervorruft. Maltose und Glucose führen zu einer Amylasefreisetzung aus den Geweben, aber Stärke hat keine Wirkung. Rinderserumalbumin (BSA), Pepton und eine Mischung von Aminosäuren haben fast keine Wirkung auf Aminopeptidase- oder Carboxypeptidasefreisetzungen, aber nur geringe Mengen von Pepton erhöhen die Freisetzung von Trypsin. Die andauernde Freisetzung von Proteasen ist ausreichend, um den Proteinbedarf für das Wachstum zu decken, und nur eine mäßige Erhöhung des Trypsins ergibt sich aus Fütterung. Kohlenhydrate werden für die Energiegewinnung verwendet, und die Freisetzung der Amylase hängt von der Menge der aufgenommenen Nahrung ab. Das Neuropeptid Allatostatin AS 5 (Grybi-AS 5) erhöht die Freisetzung von Amylase in gefütterten Weibchen, aber nicht von Trypsin oder Aminopeptidase. In ungefütterten Grillen hingegen wurde die Freisetzung von Amylase und Trypsin durch AS 5 erhöht. Gefütterte Grillen haben genügend Trypsin im Darm, um die benötigten Aminosäuren zu erhalten, aber nicht die Ungefütterten; deshalb macht die Erhöhung der Trypsin-freisetzung durch AS in ungefütterten Grillen auch Sinn.

2 . Steuerung der Enzymfreisetzung in den Larven von S. *frugiperda*. Flach-geschnittene Caecumpräparate wurden verwendet, um die Freisetzung von Amylase, Trypsin und Aminopeptidase als Antwort auf spezifische Nährstoffe im Futter und auf spezifische Neuropeptide zu testen. Die Sekretionsrate des Ventriculumepithels für

Amylase und Trypsin war ungefähr 20 % der Menge der anwesenden Enzyme im Lumen, was in Anbetracht der hoch wirksamen Gegenstromwiederverwertung der Enzyme erkennen lässt, dass die Sekretionsrate hoch genug ist, um ausscheidende Enzyme zu ersetzen. Kohlenhydrate aus der Nahrung werden für den Energiehaushalt verwendet, und die Amylaseaktivität im Darm passt sich an die Kohlenhydratzufuhr an. Die Amylaseaktivität ist 5-mal höher in gefütterten im Vergleich zu ungefütterten Larven, und Zucker im Inkubationsmedium bewirkt mehr als eine 300%ige Erhöhung der Amylasefreisetzung. Pflanzen enthalten einen verhältnismäßig niedrigen Proteinanteil, aber besonders die Larven brauchen Proteine für Wachstum. Die Larven können es sich nicht leisten, Proteine durch Ausscheidung zu verlieren. Deshalb bleibt die Trypsinaktivität sogar in ungefütterten Larven hoch. Infolgedessen haben Proteine und Aminosäuren wenig oder keine Auswirkung auf die Freisetzung von Trypsin oder Aminopeptidase in inkubierten Geweben. Die Kontrolle der Enzymfreisetzung als Antwort auf Füttern wird sehr wahrscheinlich durch Neurohormone vermittelt. Tatsächlich hemmt das Allatostatin Spfr-AS A5 die Freisetzung von Amylase und Trypsin. Allatostatin A5 hemmt auch die Myoaktivität im Ileum und Allatotropin erhöht die Myoaktivität.

3. Hemmung der Enzymfreisetzung in den Larven von S. *frugiperda*. Exogene Hemmstoffe werden von Pflanzen produziert und von den Insekten mit der Nahrung aufgenommen. Endogene Hemmstoffe werden in Darmepithelzellen gebildet. Eine dosisabhängige Hemmung der endogenen Enzyme kommt im Lumen nach der Fütterung von L6 Larven mit dem exogenen Serinproteasehemmer aus Sojabohnen (SBTI), dem spezifischen Trypsininhibitor (TLCK), einem Aminopeptidasehemmer (Bestatin), und einem Amylaseinhibitor aus Weizen vor. Hemmung in Gewebeextrakten wird nur bei höheren Dosen von SBTI und TLCK gefunden. Die Hemmung der Enzymfreisetzung ins Inkubationsmedium ist nur mit sehr hohen Dosen von SBTI sichtbar. Hemmung der Enzymaktivitäten in den Geweben und Hemmung der Enzymfreisetzung lassen eine direkte zelluläre Antwort auf einem anwesenden Hemmstoff im Lumen vermuten. Der Anstieg der Aminopeptidaseaktivität als Antwort auf den aufgenommenen Trypsininhibitor (d.h. eine Wechselwirkung) zeigt eine zelluläre Synthese als Antwort auf das Produkt eines anderen Verdauungsenzyms an. Die untersuchten Enzyme werden nach 10 min bei 90°C irreversibel inaktiviert, aber die entsprechenden Inhibitoren nicht. Nur

so konnten endogene Hemmstoffe festgestellt werden. Trypsin wird auch bei ungefütterten Larven freigesetzt und könnte dem Darmepithel schaden. Wir vermuten, dass die endogenen Proteasehemmer das Epithel in hungernden Larven schützen. Zum ersten Mal präsentieren wir eine Erklärung für die eigentlich gefährliche kontinuierliche Freisetzung von aktivem Trypsin.

List of publications

Publications in peer reviewed journals

Woodring J., Diersch S., **Lwalaba D**., Hoffmann K.H. and Meyering-Vos M. (2009): Control of the release of digestive enzymes in the caeca of the cricket *Gryllus bimacullatus*. Physiological Entomology 34, 144-151.

Lwalaba D., Hoffmann K.H. and Woodring J. (2009): Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*. Archives of Insect Biochemistry and Physiology. 73, 14-29.

Lwalaba D., Hoffmann K.H. and Woodring J. (2010): Exogenous and endogenous inhibitors of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda*. Journal of Insect Physiology, in review.

Published conference abstracts

Lwalaba D., Hoffmann K.H. and Woodring J. (2009): Control of the release of digestive enzymes in the larvae of the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae). 39th Conference of the Ecological Society of Germany, Austria and Switzerland. Bayreuth, Germany. Abstract book, p.13.

Abbreviations

AKH	adipokinetic hormone
AS	allatostatin
AT	allatotropin
BSA	bovine serum albumin
BT	*Bacillus thuringiensis*
CA	corpora allata
CCK	cholycytokininen
cDNA	complimentary deoxyribonucleic acid
Cry	crystal proteins
Grybi-AS	*Gryllus bimaculatus* allatostatin
HGR	high glucose Ringer
HPLC	high performance liquid chromatography
IGR	insect growth regulators
JH	juvenile hormone
kDa	kilo Dalton
Manse-AT	*Manduca sexta* allatotropin
mM	Millimolar
mRNA	messenger ribonucleic acid
PIs	protease inhibitors
PM	peritrophic membrane
SBTI	soybean trypsin inhibitor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Spofr-AS	*Spodoptera frugiperda* allatostatin
TAG	triacylglycerol
TLCK	N-α-tosyl-L-lysine chloromethyl ketone

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und dabei keine andern als die angegebenen Hilfsmittel verwendet habe.
Ferner erkläre ich, dass ich nicht anderweitig versucht habe, mit oder ohne Erfolg, diese oder eine gleichartige Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, den 03-01-2010

Lwalaba Digali

Erklärung

Hiermit erkläre ich, dass ich selbständig und ohne fremde Hilfe alle Experimente aus den drei Publikationen in meiner Doktorarbeit durchgeführt habe. Frau PD Dr. Martina Meyering-Vos hat mich in die Enzymkinetik eingeweiht. In die Arbeitsmethoden mit Verdauungsenzymen wurde ich zu Beginn meiner Doktorarbeit von Herrn Prof. Joseph Woodring eingewiesen, der auch meine Experimente während der Doktorarbeit überwachte.

In der Publikation 1 habe ich die Strategie für den Test der Wirkungen von Glucose, Maltose und Stärke auf Amylasefreisetzung und von BSA, Pepton und einer Aminosäuremischung auf Trypsin-, Aminopeptidase-, und Carboxypeptidase-freisetzungen in den Caeca von *Gryllus bimaculatus* selbständig durchführt. Frau Sandra Diersch hat im Rahmen ihrer Diplomarbeit die Wirkungen von *G. bimaculatus* Allatostatin 5 auf die Enzymfreisetzungen getestet. Die anderen Experimente, einschließlich der Gewebevorbereitungen, habe ich selbständig durchgeführt. Schließlich habe ich meine Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statistischen Analysen durchgeführt. Den Text des Manuskripts hat Herr Prof. Joseph Woodring vorbereitet, bevor er mit Hilfe von Frau Sandra Diersch, Herrn Prof. Dr. Klaus H. Hoffmann, Frau PD Dr. Martina Meyering-Vos und meiner Hilfe fertiggestellt wurde.

In der Publikation 2 habe ich die Strategie für die Bestimmung der Wirkungen von Nährstoffen (Glucose, Maltose, Stärke, BSA, Pepton und eine Aminosäuremischung) und Neurohormonen (*Spodoptera frugiperda* Allatostatin 5 und 6, und Manse-Allatotropin) auf die Freisetzung der Verdauungsenzyme im Darm von *S. frugiperda* Larven selbständig geplant und durchgeführt, ebenso wie die Wirkungen der Neurohormone auf die Myoaktivität im Darm. Auch die übrigen Experimente, einschließlich der Insektaufzucht und Gewebevorbereitungen, habe ich selbständig durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statistischen Analysen durchgeführt. Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Herrn Prof. Dr. Joseph Woodring und Herrn Prof. Dr. Klaus H. Hoffmann fertiggestellt wurde.

In der Publikation 3 habe ich die Untersuchungen zur Wirkung der exogenen Enzymhemmer von Trypsin- (SBTI), Aminopeptidase- (Bestatin) und α-Amylase (Weizen α-A1) auf die Larven von *Spodoptera frugiperda* selbst durchgeführt und bewertet. Die Methoden für die Bestimmung der Wirkung von endogenen Enzymhemmern habe ich selbst entworfen. Dieses Verfahren beruht auf der Tatsache, dass die Enzymaktivität durch Erhitzung (10 min bei 90^{o} C) unwiderruflich ausgeschaltet wurde und gleichzeitig die Hemmwirkung erhalten blieb. Auch die übrigen Experimente, einschließlich der Insektenaufzucht und Gewebe-vorbereitungen, habe ich selbständig durchgeführt. Schließlich habe ich alle Experimente selbständig ausgewertet, die Abbildungen angefertigt und die statischen Analysen durchgeführt. Den Text des Manuskripts habe ich selbst vorbereitet, bevor er mit Hilfe von Herrn Prof. Dr. Joseph Woodring und Herrn Prof. Dr. Klaus H. Hoffmann fertiggestellt wurde.

Bayreuth, den 03-01-2010

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