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.*

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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

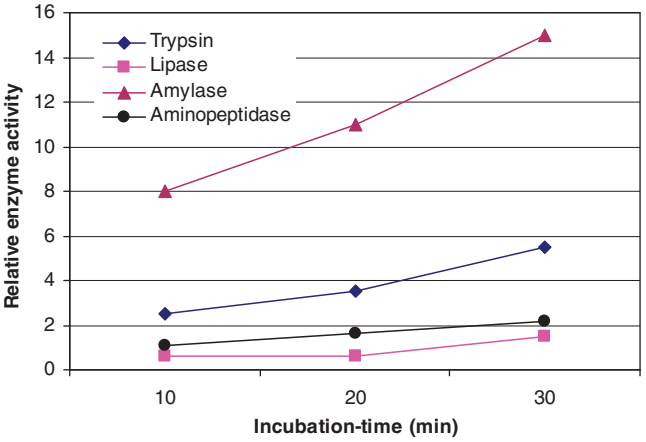

Figure 1. The rate of digestive enzyme release from the flat-sheet preparation of the ventriculus incubated in low glucose ringer (10 mg glucose/100 ml *Spodoptera* Ringer) for 30 min.

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.

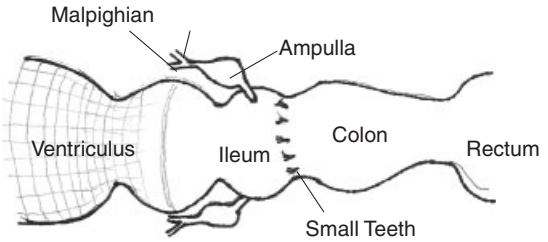

Figure 2. Schematic diagram of the hindgut of *Spodoptera frugiperda*.

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

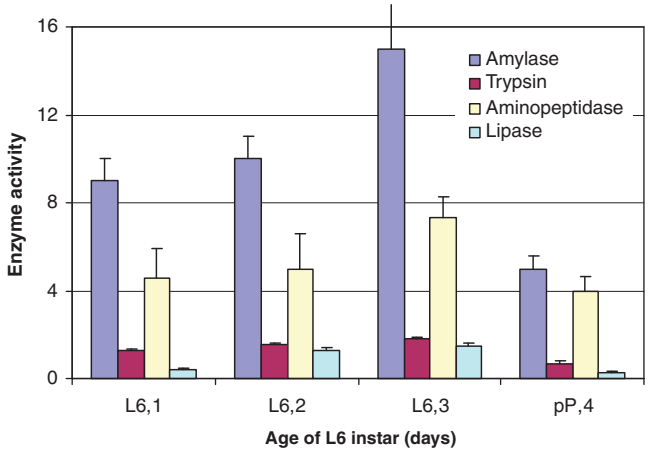

Figure 3. The release of amylase, lipase, aminopeptidase, and trypsin from the ventriculus of different aged L6 larvae (days 1–3) fed an artificial diet (Bayer CropScience, Monheim). The lipase values are multiplied by 10 (only in this figure) for ease of comparison. Units: see Materials and Methods.

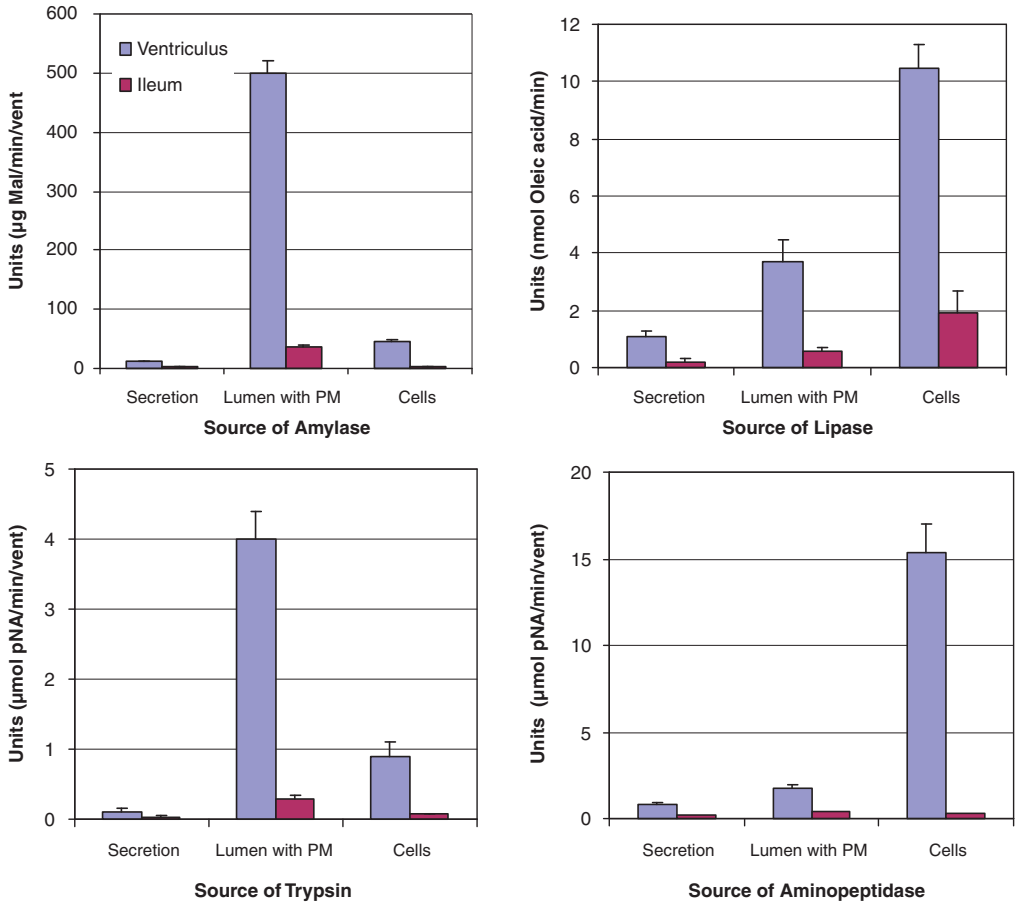

Figure 4. Amylase, lipase, trypsin, and aminopeptidase release from crop, ventriculus, and ileum of L6, day-3 larvae-fed synthetic diet.

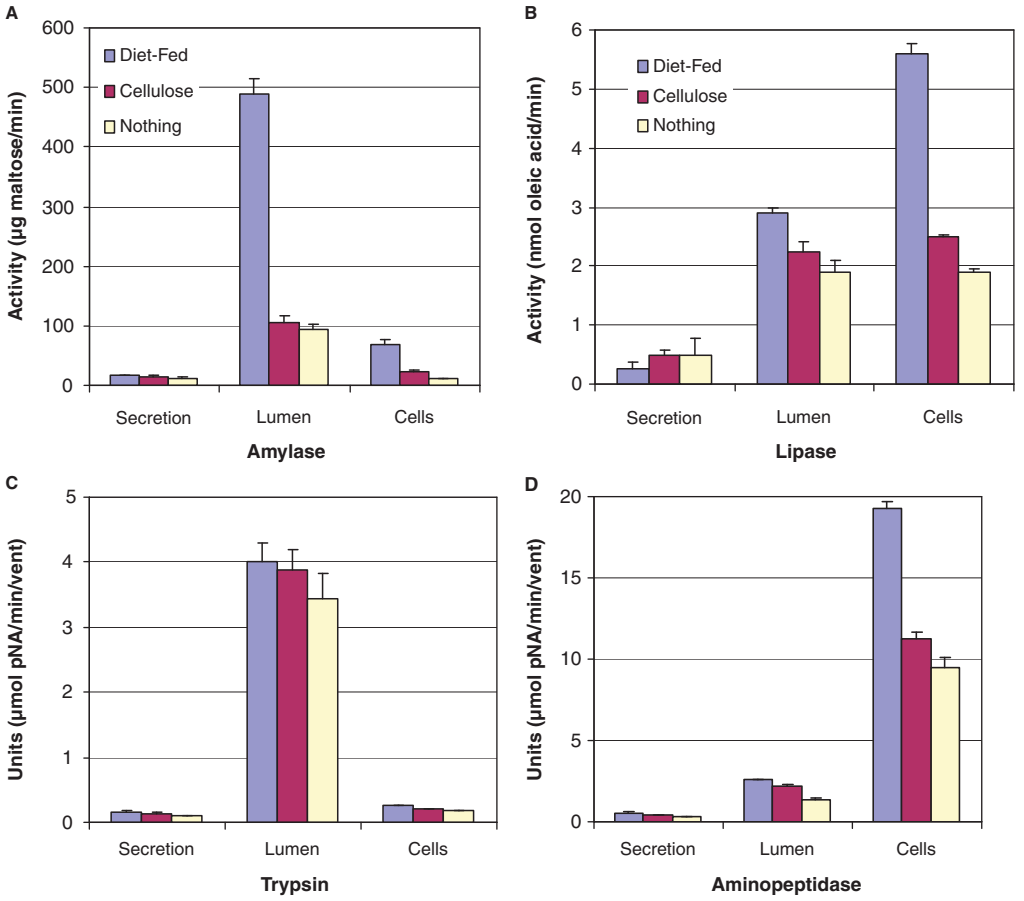

Figure 5. Amylase (**A**), lipase (**B**), trypsin (**C**), and aminopeptidase (**D**) release from the ventriculus of L6, day-3 larvae provided a synthetic diet (see Fig. 3), a cellulose diet, or nothing immediately after the moult to L6.

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

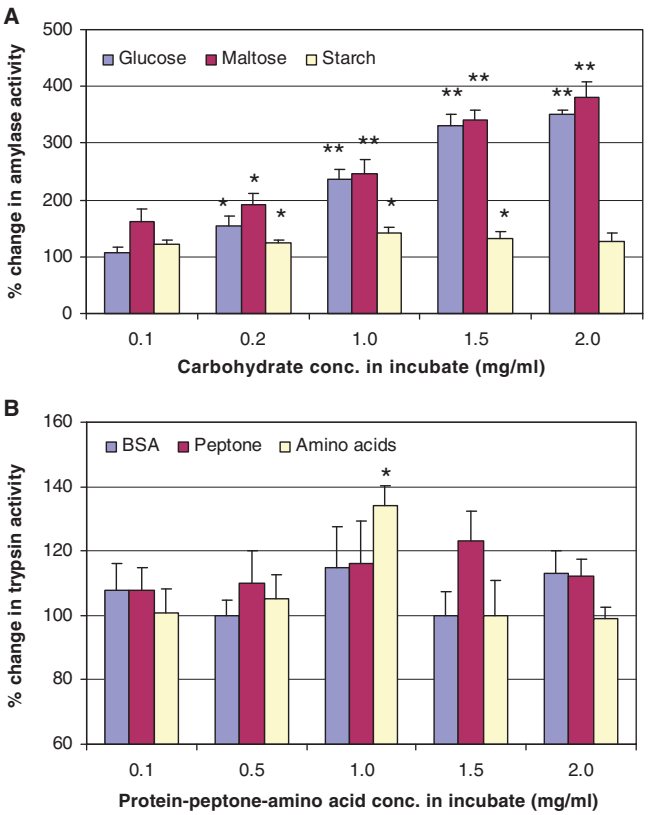

Figure 6. Effect of nutrients in the incubation medium of the ventriculus (L6, day 3) on the enzyme release (30-min incubation at 30°C). **A:** Effect of glucose, maltose, and starch on amylase release. **B:** Effect of BSA, peptone, and an amino acid mix on trypsin release. Control Ringer = 100%. Significant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

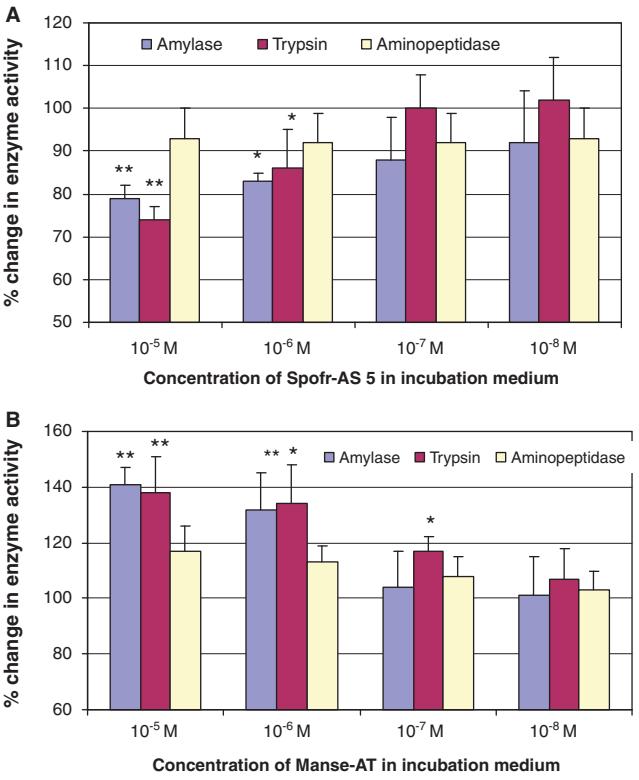

Figure 7. Effects of neuropeptides added to the incubation medium of the ventriculus (L5, day 3) on the enzyme release (30-min incubation at 30°C). **A:** Effect of the allatostatin Spofr-AS A5. **B:** Effect of the allatotropin Manse-AT. Control Ringer = 100%. Significant increase or decrease: * > 0.05,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