ABSTRACT

JEFFERS, LAURA ANN. The Movement of Proteins across the Digestive System of Tobacco Budworm, *Heliothis virescens*. (Under the direction of R. Michael Roe.)

Bovine serum albumin (BSA) and anti-BSA polyclonal antibody were used as model polypeptides to examine protein movement across the insect digestive system and cuticle and their accumulation in hemolymph of fourth stadium tobacco budworms, *Heliothis virescens*. The use of hydrateable meal pads to deliver a specific concentration of these two proteins in insect diet was investigated. Continuous feeding on artificial diet containing 0.8 mg of anti-BSA/g hydrated diet resulted in 2430±125 and 3459±105 ng of anti-BSA/mL hemolymph after 8 and 16 h, respectively (average ± 1 SEM), as determined by ELISA. Continuous feeding on meal pads with the same concentration of BSA resulted in 1547±132 and 1623±122 ng of BSA/mL hemolymph at 8 and 16 h, respectively. No BSA or anti-BSA was found in the feces, and when 5 μg of these two proteins were applied topically in DMSO to the cuticle, neither protein was found in the hemolymph after 4 h. Western blot analyses using native and/or de-naturing gel electrophoresis demonstrated that both BSA and anti-BSA were not degraded in the hydrated meal pads and were also unchanged in the hemolymph, retaining the multimeric structure for BSA and the antigen reactivity for anti-BSA. When 1 μg of anti-BSA or BSA was injected into the hemocoel of fourth instars, the concentrations decreased with time and 120 min after injection were 0.6 and 20% of the original concentration, respectively. When added at the same original concentration to hemolymph in vitro, the decrease was 81.5 and 57.5%, respectively, at 120 min. Apparently, the accumulation of native anti-BSA and BSA protein in insect hemolymph is the result of the rate of their transfer from the diet versus their rate of turnover in the hemolymph. Hemolymph turnover of these proteins appears to be the result of degradation and sequestration.

THE MOVEMENT OF PROTEINS ACROSS THE DIGESTIVE SYSTEM OF TOBACCO BUDWORM, *HELIOTHIS VIRESCENS*

by
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BIOGRAPHY

Laura Ann Jeffers was a Century Scholarship Recipient at the University of North Carolina at Greensboro. After two years Ms. Jeffers transferred to North Carolina State University. She completed her Bachelor of Arts in Chemistry in 1997 and her Bachelor of Science in Biochemistry in 2000.

Ms. Jeffers became interested in Entomology while working at Aventis Crop Science (now Bayer Crop Science). Under the guidance of Dr. Vincent Salgado, Ms. Jeffers was responsible for the initial mode of action testing for the New Leads Team.

Ms. Jeffers came to work with Dr. R. Michael Roe at Dearstyne in 2000. She primarily works with protein movement across the digestive system in *Heliothis virescens* and protein delivery techniques as a novel approach to insect control. In 2003 she was awarded a NIH/NCSU Biotechnology Training Fellowship to continue her work with protein delivery.

Ms. Jeffers enjoys traveling, a passion she inherited from her parents, Jim and Grace Jeffers. She also enjoys gardening, stained glass, and spoiling her pound dogs, Ralph and JoJo.

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For *Journal of Insect Physiology*

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Review: The movement of proteins across the insect digestive system

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Abstract

In comparison to mammals, our understanding of protein movement across the digestive system of insects is minimal. It has been documented that a small fraction of ingested proteins pass intact into the hemolymph across the digestive system in insects and ticks. Examples of protein movement across the insect orders Diptera, Hemiptera, Siphonaptera, and Lepidoptera, as well two tick families Ixodidae and Argasidae are discussed. The mechanisms by which these large macromolecules move across the digestive system are unknown. Proteins have been shown to diffuse across the midgut epithelial cells after feeding.

1. Introduction

Following the successful use of the protein delta-endotoxin from *Bacillus thuringiensis* (*Bt*) as an insecticide (Gill et al., 1992; Fischhoff, 1996; Gould, 1998), a number of proteins and polypeptides have been proposed for their potential use as insecticides. Some of these are juvenile hormone esterase (Roe and Venkatesh, 1990; Hammock and Philpott, 1992), trypsin modulating oostatic factor (TMOF) (Borovsky et al., 1990), the mite toxin TxP-I (Tomalski et al., 1988; Tomalski et al., 1989), and the scorpion toxins AaIt (Zlotkin et al., 1971; Zlotkin et al., 2000), $LqhIT_3$ (Zlotkin et al., 1991), and $BotIT_2$ (Stankiewicz et al., 1996). A major obstacle to overcome in using the majority of protein toxins as insecticides is delivery to the target site are across the digestive system. Oral delivery of protein insecticides is made difficult due to their

degradation by digestive endo- and exo-peptidases and their limited movement across the gut epithelium. One of the major reasons the *Bt* delta-endotoxin has been so successful is it acts directly on the lining of the digestive system rather than requiring export into the insect body. One method that has been investigated to circumvent the delivery problem has been the use of transgenic baculoviruses. Baculoviruses are safe and selective, but this system is slow acting, expensive and unstable in the environment (Duffey et al., 1995). A great concern with this approach is the public acceptance of the wide-spread release of transgenic viruses.

2. Protein movement across the insect and tick digestive system

Wigglesworth (1943) was one of the first researchers to demonstrate the passage of intact proteins across the insect digestive system. He found that a small amount of ingested hemoglobin is absorbed across the gut of *Rhodnius prolixus* into the hemolymph. Since then numerous blood feeding arthropods have been shown to have intact host proteins in their hemolymph after feeding. Wigglesworth (1943) was one of the first researchers to demonstrate the passage of intact proteins across the insect digestive system. He found that a small amount of ingested hemoglobin is absorbed across the gut of *Rhodnius prolixus* into the hemolymph. Since then numerous blood feeding insects and ticks as well as a few nonbloodfeeding insects have been shown to have intact host proteins in their hemolymph after feeding (table 1).

2.1 Bloodfeeding insects

The following blood feeding insects have been found to have intact host proteins in their hemolymph after feeding: the buffalo fly, *Haematobia irritans* (Allingham et al., 1992); the tsetse fly, *Glossina morsitans* (Nogge and Giannetti, 1979; Modespacher et al., 1986); the flesh fly, *Sarcophaga falculata* (Fishman and Zlotkin, 1984); the mosquitoes, *Aedes aegypti* (Hatfield, 1988), *Anopheles gambiae* (Vaughan and Azad, 1988), *Anopheles albimanus* (Vaughan and Azad, 1988), *Anopheles stephensi* (Schneider et al, 1986; Vaughan and Azad, 1988), and *Culex pipiens* (Vaughan and Azad, 1988); and the cat flea, *Ctenocephalides felis* (Vaughan et al., 1998)

Nogge and Giannetti (1979) demonstrated that intact human albumin and fragments of immunoglobulins are found in the hemolymph of *Glossina morsitans,* the tsetse fly, after feeding on human blood. The presence of serum proteins in the hemolymph of *G. morsitans* after oral administration was determined by immunodiffusion tests conducted in agar gel using the method of Schneweis and Nahmias (1971). Collected hemolymph was allowed to diffuse against anti-albumin IgG, anti-human IgG (complete) and anti-human IgG (Fab fragments). No intact IgG was detected in the hemolymph, but Fab fragments and albumin were present. Nogge and Giannetti found that about 0.035% of albumin added to a bloodmeal reached the hemolymph unchanged. Passage of the the IgG (complete) was impeded by the low permeability of the peritrophic membrane, which limits the movement of compounds greater than a molecular weight of 45,000 Da (Peters and Nogge, 1980).

Using oral toxicity assays, column chromatography, and microscopic autoradiography of the native and radioiodinated toxin, the neurotoxic scorpion venom polypeptide, AaIt, was shown to cross the gut of *Sarcophaga falculata*. At 10 µg/ 100mg

of body weight, AaIt was shown to induce paralysis of flies within 1-2 h after oral administration. Oral toxicity was shown to be 0.14% of toxicity by injection. Five percent of the ingested radioactivity appeared in the hemolmyph 85 minutes after feeding flies with AaIt. Most of this polypeptide was degraded, but included 0.3% of intact toxin (Zlotkin et al., 1992).

Using ELISA and Western blots, Allingham et al. (1991) were able to detect intact host IgG in the hemolymph of blood-fed buffalo flies, *Haematobia irritans*. Heparinized ovine or bovine blood was fed to *H. irritans* continuously or for a single 10 minute period. A single protein, an uncharacterized bovine IgG or molecular weight 150 kDa, was detected in the hemolymph of blood fed flies. Because it co-migrated with bovine IgG, the hemolymph protein was apparently intact. Ninety four percent of flies observed to take a blood meal had detectable amounts of IgG in the hemolymph after feeding. The concentration of IgG in pooled hemolymph of flies allowed continuous access to blood meal ranged from 2.1-7.3 μg/mL hemolymph.

Vaughan and Azad (1988) found anti-*Rickettsia typhi* antibody was present in four species of mosquitoes after feeding on rats immunized with a *R typi* extract. Mosquitoes were fed on immune rat (IFA (indirect immunofluorescent assay) titer ≥ 1:6,400) or control groups fed on a nonimmune rat (IFA titer <1:40). Hemolymph was collected by hemocoel perfusion (Vaughan et al., 1988), and an IFA was used for the detection of anti-*R typhi*. Two controls were designed to ensure the digestive tract remained intact and hemolymph collection was not contaminated by the contents of the bloodmeal. First, mosquitoes were fed a sucrose solution containing methylene blue before hemocoel perfusion, demonstrating that perfusion did not rupture digestive tract.

Second, trials were completed using alternate methods of hemolymph collection. Mosquito legs were amputated at the coxa-trochanter joint and hemolymph was collected in a droplet of saline and were found comparable to samples using hemocoel perfusion. Anti-*R typhi* was present in the hemolymph of *Anopheles stephensi* at 3, 6, and 18 h after feeding, *An. gambiae* at 3, 6, 18, and 24 h after feeding, *An. albimanus* at 3, 6, 18, and 24 h after feeding, and *Culex pipiens* at 3 h after feeding.

Siphonaptera

Vaughan et al (1998) fed heparinized cat blood by membrane feeders to the cat flea, *Ctenocephalides felis*. Using ELISA and Western blots, cat IgG was found to be present in hemolymph of engorged female fleas 1 h after ingestion 35 ± 14 μg/mL. Following a single blood meal 100% of both male and female fleas had detectable cat IgG in their hemolymph 1 h after feeding. Following a single blood meal, cat IgG was only present in 50% of the flea hemolymph tested at 3 h after ingestion, and cat IgG was only detectable in 10% of samples at 18 h after ingestion. Fleas were also allowed access to blood over a 72 h period, and the cat IgG concentration remained fairly constant (3-16 pg per sample) over the entire period.

2.2 Ticks

The movement of intact proteins across the tick digestive system has been shown in the following Argasidae: *Ornithodoros moubata* (Chinzei and Minoura, 1987; Ben-Yakir, 1989); and Ioxidae: *Amblyomma americanum* (Jasinskas et al., 2000), *Hyalomma*

excavatum (Ben-Yakir, 1989), *Dermacentor variabilis* (Ackerman et al., 1981), and *Rhipicephalus sanguineus* (Ben-Yakir, 1989).

2.2.1 Argasidae

Chinzei and Minoura (1987) studied the antigenicity and concentration of detected IgG in the hemolymph of *Ornithodoros moubata. O. moubata* were allowed to feed 1-2 h on blood containing either rabbit IgG or human IgG through an artificial membrane. Hemolymph was collected from female ticks 7 days after feeding and applied to Ouchterlony plates. The hemolymph of ticks engorged on rabbit IgG reacted with anti-rabbit IgG to form an immunoprecipitin line with the preciptin line between rabbit IgG and anti-rabbit IgG. Similarly the hemolymph of ticks engorged on human IgG reacted with anti-human IgG to form an immunoprecipitin. These observations demonstrate that IgG in the tick hemolymph is immunologically identical to the IgG found in the blood meal, suggesting female ticks directly absorb host IgG through the gut wall into the hemocoel without loss of IgG antigenicity. To determine the change of IgG titer in tick hemolymph after feeding a single radical immunodiffusion test was used. After feeding (day 1-5 after feeding) the IgG levels were very low (< 1 ng/ μL) followed by a sudden increase 5-7 days after feeding (approximately 10-15 ng/ μL).

2.2.2 Ixodidae

Jasinskas et al (2000) used capillary feeding to introduce compounds into the midgut of adult female *Amblyomma americanum*. The proteins studied were mouse, donkey, and human IgG, mouse serum albumin, and chloramphenicol acetyltransferase

(labeled with ^{125}I, ^{14}C, or biotin). The entry of the immunoglobulins into the hemolymph was greater (6%) after 6 h than the smaller proteins chloramphenicol acetyltransferase and albumin (3% and 1%, respectively). Comparing the uptake of different types of IgG in the hemolymph, these results indicate uptake is not specific for mouse, donkey, or human IgG.

In 1989 Ben-Yakir measured host IgG concentration in the hemolymph of female hard and soft ticks. *Hyalomma excavatum, Rhipicephalus sanguineus, Ornithodoros tholozani, and O. moubata* were allowed to feed on rabbits that had been immunized with ovalbumin. *Argas persicus* were allowed to feed on a chicken that had been immunized with cytochrome C. Hemolymph samples were taken 24 h after removal from their hosts. Although host IgG was not detected in the hemolymph *of O. tholozani* or *A. persicus,* host IgG was found in the hemolymph of *H. excavatum*, *R. sanguineus*, and *O. moubata* (7, 5, and 0.15 μg/mL, respectively). Using Protein A, the host IgG found in hemolymph was shown to be intact in all three species (Ben-Yakir, 1989). After being parasitized, it is possible for a host to develop resistance to tick engorgement, leading to fewer ticks engorging and less blood ingested. The ticks that feed on resistant hosts can show slowed development, decreased fecundity, and increased mortality (Ben-Yakir, 1989).

2.3 Nonbloodfeeding insects

In contrast to the hematophagous insects and ticks, there have fewer studies on non-hematophagous insects including the silkworm, *Bombyx mori* (Hirayama et al., 2000); the western tarnished plant bug, *Lygus hesperus* (Habibi et al., 2002); the

European corn borer, *Ostrinia nubalilalis* (Ben-Yakir and Shochat, 1996), and the tobacco budworm, *Heliothis virescens* (Jeffers, unpublished).

2.3.1 Lepidoptera

Ben-Yakir and Shochat (1996) studied the fate of ingested anti-ovalbumin IgG in European corn borer (ECB). The ECB larvae were fed on a serum-containing diet between 12 and 96 h. The concentration of anti-ovalbumin in the diet was 1,200 μg/g. After 48 h of feeding on the anti-ovalbumin diet the concentration of the IgG in the midgut was found to be 673 ± 447 μg/g (average ± SD). The larvae were removed from the anti-ovalbumin diet and the concentration of the IgG was determined to be 204 ± 153 μg/g and 64 ± 38 μg/g at 6 and 18 h post feeding respectively. The concentration of anti-ovalbumin in the hemolymph of the ECB was 2.40 ± 0.98 μg/ml, 2.06 ± 1.14 μg/ml, and 1.64 ± 1.32 μg/ml after 24, 48, and 96 h of feeding on the IgG diet. The concentration of IgG in the hemolymph at 24, 48, and 96 h was 1/500 of that in the diet. The protein concentration in the hemolymph was directly related to the antibody titer in the diet.

Mulberry leaf urease was found to pass through the gut wall of the silkworm, *Bombyx mori*, into the hemolymph without being digested (Hirayama et al., 2000). When silkworms feed on mulberry leaves, urease activity is detected in the hemolymph from the beginning of spinning to the pharate adult stage. Silkworms feeding on an artificial diet (contains no urease) do not have detectable urease activity (Yamada et al., 1984). To determine if the hemolymph urease and leaf urease were identical, urease was purified from the hemolymph of the spinning larvae reared on mulberry leaves and compared to mulberry leaf urease. Both the hemolymph urease and the mulberry urease co-migrated

with apparent molecular weight of 90.5 kDa on SDS-PAGE gels. The hemolymph urease cross reacted equally with the mulberry urease using four out of six monoclonal antibodies raised against jack bean seed urease. The K_m value for urea and the optimum pH for activity were almost the same for the two enzymes. Comparing the sequence of the first 20 amino terminal amino acids, the hemolymph urease was identical to the mulberry leaf urease (Hirayama et al., 2000).

Bovine serum albumin (BSA) and anti-bovine serum albumin (anti-BSA), were used to determine rate of absorption, passage, and fate of proteins in 4th instar tobacco bud worms, *Heliothis virescens*. After 8 or 16 h of feeding on diet containing anti-BSA the concentration of anti-BSA in larval hemolymph was 2430 ± 125 ng/mL (average ± 1 SEM) or 3459 ± 105 ng/mL respectively. After 8 or 16 h of feeding on BSA containing diet the concentration of BSA in larval hemolymph was 1547 ± 132 ng/mL or 1623 ± 122 ng/mL respectively (all values given are the average ± 1 standard error of the mean). Rinse and topical bioassays were designed to eliminate routes by which a measurable amount of anti-BSA or BSA could enter into the hemolymph during feeding, showing all protein detected in the hemolymph is from passage across the gut. Using Western blots, all three multi-meric forms of BSA were in the hemolymph after feeding in the same ratios as found in the diet. The light and heavy chains of anti-BSA were also found in the same ratios in the diet as in the hemolymph after feeding. *In vivo* injections and *in vitro* metabolism studies determine anti-BSA and BSA behavior in larval hemolymph and pooled plasma. In the hemolyph the concentration of anti-BSA or BSA had decreased by 99.4% or 80.0% after 120 min respectively. In the pooled plasma the concentration of

anti-BSA or BSA decreased by 18.5% or 42.5% after an incubation of 120 min respectively (Jeffers, unpublished).

2.3.2 Hemiptera

Casein (a digestible protein) and Green Fluoroescent Protein (GFP, a non-digestible protein) were fed to the western tarnished plant bug, *Lygus hesperus*, to study passage and absorption. Intact FITC-casein (fluorescein isocyanate conjugated) was detected in the hemolymph at 2 and 4 h after feeding on a sachet system containing artificial diet and 1% FITC-casein and then moved to control diet after 2 h. At 12 h no FITC-casein was detected in any tissue. Intact GFP was detected in the hemolymph at 3 and 6 h after feeding on a sachet system containing artificial diet and 0.05% GFP and then moved to a control diet after 3 h. At 12 h no GFP was detected in the hemolymph or gut by Western blot. The ratio of the GFP signal in the hemolymph to the signal in the gut was greater at 6 h than 3 h (Habibi et al., 2002).

3. Possible Mechanisms

The transfer of intact proteins across the digestive system and into the circulatory system of mammals was documented as early as 1936 by Verzár and McDougall. Macromolecular (including protein) transport across the mammalian digestive system has been discussed in the following reviews: Silk (1980), Udall and Walker (1982), Silk and Keohane (1983), Gardner (1984, 1988), Weiner (1988), and Pácha (2000). The transport of macromolecules across the stomach and small intestinal wall is important during early

postnatal life because it facilitates the absorption of growth factors, antigens, and immunoglobulin from maternal colostrum and milk. In early mammalian development the transport of macromolecules occurs in enterocytes and follows two pathways: specific receptor-mediated transcytosis and nonspecific transcytosis (Pácha, 2000). In specific receptor-mediated transepithelial transport of immunoglobulins the IgG present in milk (or colostrum) bind to a specific receptor in the apical membrane, the complex is endocytosed, moved through the cell within a transport vesicle, and secreted into the contraluminal compartment. In some mammals, it possible to observe a massive absorption of IgG via a nonselective endocytotic pathway when intraluminal macromolecules are endocytosed, partially destroyed, and partially transported across the enterocytes.

In comparison to mammals, very little is known about the movement of proteins across the insect gut. Hatfield (1988) found mouse IgG persisted in *Aedes aegypti* mosquito bloodmeals for 2-3 days after ingestion. Immunoenzyme labeling showed that mouse IgG bound to the midgut epithelium of *A. aegypti* after feeding. Immunogold labeling of thin sections showed mouse IgG in the cytoplasm of the midgut epithelium microvilli. Finally, mouse IgG was found in the mosquito hemolymph using an ELISA. The IgG was not found to be bound to any other tissue besides the gut. Hatfield proposed that IgG might actively and selectively move through the midgut epithelium similar to the mammalian mechanisms described earlier. Alternatively, IgG may be passively transferred of between the epithelial cells, a "leaky" midgut, the mechanism by which arboviruses appear in a bloodmeal after feeding (Boorman, 1960; Miles et al., 1973; Hardy et al., 1983).

Using electron microscopy and the application of electron-dense tracers together with ingested food, Modespacher et al. (1986), wanted to determine where and when intact proteins are moved across after blood uptake. After feeding with human serum, horseradish peroxidase (HRP) moves into the intracellular clefts of the anterior-part and middle-part (0.5 and 1 h after feeding respectively) of the midgut in the tsetse fly. One h after feeding HRP was present in basal labyrinth of the midgut epithelium in the anterior and middle-parts. After 2 h most basal parts of the basal labyrinth were preferentially labeled as well as the basal lamina, indicating HRP reached the hemolymph. No HRP was found in the posterior-part of the midgut, and the authors found no indication that pinocytosis is the mode of transport.

Fishman and Zlotkin (1984) introduced HRP to the flesh fly, *Sarcophaga falculata,* (0.03-0.3 mg per insect) and the movement of HRP was followed by light and electron microscopy across the midgut. After crossing the peritrophic membrane and spreading into the intercellular spaces of the midgut epithelium, it was shown that HRP entered the epithelial gut cells of *S. faculata* through microvilli 30 min after feeding. Next HRP diffuses across the cytoplasm of the cell. Thirty to 60 min after oral application, 40% of the sections from the midgut demonstrated the absence of HRP in the apical region of the midgut epithelial cells and the accumulation of HRP at the basal regions. Finally the HRP was shown to cross the inner plasma membrane of the midgut epithelials, the basal membrane, and the layer of connective tissue covering the muscular system of the gut. The continuous diffusion throughout the cytoplasm indicated that pinocytotic uptake or vesicular transport, common in mammalian systems, was unlikely.

4. Future research directions

Recently significant advances have been made in the pharmaceutical industry for the delivery of proteins across the digestive system of humans for the treatment of diseases. One approach is the development of covalently bound protein polymers, which increase transport across biological membranes and decrease proteolytic degradation (Zalipsky and Harris, 1997; Veronese, 2001; Roberts et al., 2002; Harris and Veronese, 2002; Yowell and Blackwell, 2002). Related technology was recently developed by R. M. Roe (patent pending) for insect control. In order to apply this new technology, we must have a understanding of how insects transport proteins into the hemolymph, and therefore we herein review the previous studies on non-enhanced movement of proteins across the insect and tick digestive system.

In conclusion, protein movement across the digestive system of insects is an understudied area of research. The specific mechanisms by which proteins are able to move intact across the digestive system have yet to be found. It is difficult to compare the aforementioned studies due to the differences in species and methodology. However it seems probable that immunoglobulins are found at higher ratios than other proteins in the insect and tick hemolymph after feeding. Insects and ticks may have unique mechanisms for transporting different types of proteins across the digestive system.

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TABLES AND TABLE CAPTIONS

Table 1. Protein movement across the insect and tick digestive system. The following proteins have been shown to move intact across the digestive system of an insects or ticks.

Table 1 Species	Protein(s)	Detection Method(s)	Reference(s)
Bloodfeeding Insects			
Diptera:			
Aedes aegypti	mouse anti-BSA	ELISA[a]	Hatfield, 1988
Anopheles albimanus	rat IgG	IFA[b]	Vaughan and Azad, 1988
Anopheles gambiae	rat IgG	IFA	Vaughan and Azad, 1988
Anopheles stephensi	rat IgG	IFA	Vaughan and Azad, 1988
Culex pipiens	rat IgG	IFA	Vaughan and Azad, 1988
Glossina morsitans	albumin horseradish peroxidase	immunodiffusion tests electron microscopy	Nogge, 1979; Modespacher et al., 1986
Haematobia irritans	bovine, ovine IgG	ELISA Western blot	Allingham et al., 1992
Sarcophaga falculata	cobra neurotoxin scorpion toxin, AaIT horseradish peroxidase	toxicity microscopic autoradiography column chromatography electron microscopy	Fishman and Zlotkin, 1984; Primor and Zlotkin, 1980 Zlotkin et al., 1992
Hemiptera:			
Rhodnius prolixus	hemoglobin	spectroscopic protein assay	Wigglesworth, 1943
Siphonaptera:			
Ctenocephalides felis	cat IgG	ELISA Western blot	Vaughan et al., 1998;
Bloodfeeding Acari			
Argasidae:			
Ornithodoros moubata	human, rabbit IgG rabbit anti-ovalbumin	EIA[c] radioimmunoassay	Chinzei and Minoura, 1987; Ben-Yakir, 1989
Ixodidae:			
Amblyomma americanum	mouse, donkey, human IgG mouse serum albumin chloramphenicol acetyltransferase	ELISA PAGE and autoradiography	Jasinskas et al., 2000
Dermacentor variabilis	rabbit IgG	radioimmunoassay	Ackerman et al., 1981
Hyalomma excavatum	rabbit anti-ovalbumin	radioimmunoassay	Ben-Yakir, 1989
Rhipicephalus sanguineus	rabbit anti-ovalbumin	radioimmunoassay	Ben-Yakir, 1989

Table 1 (continued)

Species	Protein(s)	Detection Method(s)	Reference(s)
Nonbloodfeeding Insects			
Hemiptera:			
Lygus hesperus	casein green fluorescent protein	immunodetection immunohistochemical Western blot	Habibi et al., 2002
Lepidoptera:			
Bombyx mori	urease	urease protein assay Western blot	Hirayama et al., 2000
Heliothis virescens	rabbit anti-BSA BSA	ELISA Western blot	Jeffers, unpublished
Ostrinia nubalilalis	rabbit anti-ovalbumin	ELISA	Ben-Yakir and Shochat, 1996

[a] ELISA, Enzyme linked immosorbent assay,
[b] IFA, Indirect immunofluorescent assay
[c] EIA, Enzyme immunoassay

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The Movement and Fate of Proteins across the Digestive System of Tobacco Budworm, *Heliothis virescens*

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ABSTRACT

Bovine serum albumin (BSA) and anti-BSA polyclonal antibody were used as model polypeptides to examine protein movement across the insect digestive system and cuticle and their accumulation in hemolymph of fourth stadium tobacco budworms, *Heliothis virescens*. The use of hydrateable meal pads to deliver a specific concentration of these two proteins in insect diet was investigated. Continuous feeding on artificial diet containing 0.8 mg of anti-BSA/g hydrated diet resulted in 2430±125 and 3459±105 ng of anti-BSA/mL hemolymph after 8 and 16 h, respectively (average ± 1 SEM), as determined by ELISA. Continuous feeding on meal pads with the same concentration of BSA resulted in 1547±132 and 1623±122 ng of BSA/mL hemolymph at 8 and 16 h, respectively. No BSA or anti-BSA was found in the feces, and when 5 μg of these two proteins were applied topically in DMSO to the cuticle, neither protein was found in the hemolymph after 4 h. Western blot analyses using native and/or de-naturing gel electrophoresis demonstrated that both BSA and anti-BSA were not degraded in the hydrated meal pads and were also unchanged in the hemolymph, retaining the multimeric structure for BSA and the antigen reactivity for anti-BSA. When 1 μg of anti-BSA or BSA was injected into the hemocoel of fourth instars, the concentrations decreased with time and 120 min after injection were 0.6 and 20% of the original concentration, respectively. When added at the same original concentration to hemolymph in vitro, the decrease was 81.5 and 57.5%, respectively, at 120 min. Apparently, the accumulation of native anti-BSA and BSA protein in insect hemolymph is the result of the rate of their transfer from the diet versus their rate of turnover in the hemolymph. Hemolymph turnover of these proteins appears to be the result of degradation and sequestration.

INTRODUCTION

The use of the protein delta-endotoxin from *Bacillus thuringiensis* (Bt) as an insecticide, either as a spray or in transgenic crops, has revolutionized our current thinking about pesticide development and insect control. The Bt endotoxin acts directly on the midgut epithelium disrupting digestive function and ultimately causing death (Gill et al., 1992; Fischhoff, 1996). Due to its success and selectivity, many crops are now genetically engineered to express the Bt endotoxin, including cotton, maize, tobacco, and potatoes (Gould, 1998; Gill et al., 1992). With the rising public concern over transgenic crops and resistance to the Bt toxins (Tabashnik et al., 1990; Tabashnik, 1994; Alstad and Andow, 1995; Bauer, 1995; Gould et al., 1997; Huang et al., 1999), alternative insecticidal proteins are needed.

A number of polypeptides with potential use as an insecticide have been discovered, including juvenile hormone esterase (Roe and Venkatesh, 1990; Hammock and Philpott, 1992), trypsin modulating oostatic factor (TMOF) (Borovsky et al., 1990), the mite toxin TxP-I (Tomalski et al., 1988; Tomalski et al., 1989), and the scorpion toxins Aait (Zlotkin et al., 1971; Zlotkin et al., 2000), $LqhIT_3$ (Zlotkin et al., 1991), and $BotIT_2$ (Stankiewicz et al., 1996). A major obstacle to overcome in using protein toxins as insecticides that do not act directly on the lining of the digestive system is their delivery to the target site in the insect hemocoel. Oral delivery of protein insecticides is made difficult due to their degradation by digestive endo- and exo-peptidases and their limited movement across the gut epithelium. One method that has been investigated to circumvent the delivery problem has been the use of transgenic baculoviruses. Even

though baculoviruses are safe and selective (Duffey et al., 1995), this system is slow acting, expensive and unstable in the environment (Duffey et al., 1995). However, one of the greatest concerns with this approach is the public acceptance of the wide-spread release of transgenic viruses.

Wigglesworth (1943) was one of the first researchers to demonstrate the passage of intact proteins across the insect digestive system. He found that a small amount of ingested hemoglobin is absorbed across the gut of *Rhodnius prolixus* into the hemolymph. Since then numerous blood feeding arthropods have been shown to have intact host proteins in their hemolymph after feeding: the buffalo fly, *Haematobia irritans* (Allingham et al., 1992); the tsetse fly, *Glossina morsitans* (Nogge and Giannetti, 1979; Modespacher et al., 1986); the flesh fly, *Sarcophaga falculata* (Fishman and Zlotkin, 1984); the mosquitoes, *Aedes aegypti* (Hatfield, 1988), *Anopheles gambiae* (Vaughan and Azad, 1988), *Anopheles albimanus* (Vaughan and Azad, 1988), *Anopheles stephensi* (Schneider et al, 1986; Vaughan and Azad, 1988), and *Culex pipiens* (Vaughan and Azad, 1988); the cat flea, *Ctenocephalides felis* (Vaughan et al., 1998); and the ticks, *Ornithodoros moubata* (Chinzei and Minoura, 1987; Ben-Yakir, 1989), *Amblyomma americanum* (Jasinskas et al., 2000), *Hyalomma excavatum* (Ben-Yakir, 1989), *Dermacentor variabilis* (Ackerman et al., 1981), and *Rhipicephalus sanguineus* (Ben-Yakir, 1989). In contrast, there have fewer studies on non-hematophagous insects: the silkworm, *Bombyx mori* (Hirayama et al., 2000); the western tarnished plant bug, *Lygus hesperus* (Habibi et al., 2002); and the European corn borer, *Ostrinia nubalilalis* (Ben-Yakir and Shochat, 1996).

In the past 2 decades, significant advances have been made in the pharmaceutical industry for the delivery of protein across the digestive system of animals and humans for the treatment of diseases. One approach is the development of protein polymers (Zalipsky and Harris, 1997; Veronese, 2001; Roberts et al., 2002; Harris and Veronese, 2002; Yowell and Blackwell, 2002). Related technology was recently developed by Roe (patent pending) for insect control. However, compared to animal and humans, the study of protein movement across the digestive system of insects is an understudied area of research. In addition, most of the available literature in this area has focused on blood feeding arthropods. In the current study, we examined the movement of BSA and anti-BSA antibody as model proteins across the digestive system of an important agricultural pest, the tobacco budworm (*Heliothis virescens*) and examine their stability during digestion and in hemolymph.

MATERIALS AND METHODS

Insects

Tobacco budworms*, Heliothis virescens,* were obtained as third instars on artificial diet (Burton, 1970) in 1-oz plastic cups (Solo, Woodridge, IL) from the Department of Entomology at North Carolina State University (Raleigh, NC). The budworm strain is HV97, which was established from field collections from tobacco plants in North Carolina in 1996 and 1997. For the studies that follow, larvae (45-55 mg) that had completed their feeding in the third stadium were removed from the diet and placed individually into 1-oz Solo cups without diet. The cups were sealed with a paper lid. The larvae were then maintained at 27±1°C, 60±10% relative humidity, and a 14:10 light:dark

cycle for 6 h. Following this treatment, only larvae that had completed ecdysis to the fourth stadium as determined by the presence of a shed, third-stadium head capsule were used in the studies that follow.

Feeding Bioassay

Solo cups containing approximately 2 mL of artificial diet (Burton, 1970) were frozen at -80° C for 48 h and then lyophilized on a Virtis Bench Top 6 freeze-dryer (Virtis, Gardiner, NY; cold trap = -70°C, ≈200 mTorr, ambient temperature = 23°C) for 48 h (Roe et al., 1999; Roe et al 2000; Bailey et al., 2001). The lyophilized diet was then divided with a razor blade into 80-120 mg meal pads, which were stored in the dark at room temperature and 0% humidity until needed.

For the feeding assay, the 80-120 mg lyophilized meal pads were placed into a 1-oz plastic cup (1 per cup) and hydrated with a 1.0 mg/mL solution of rabbit (polyclonal) anti-Bovine Serum Albumin (anti-BSA, Sigma, Saint Louis, MO) in PBST (Dulbecco's Phosphate Buffering Solution, pH 7.4, 0.008 M sodium phosphate, 0.002 M potassium phosphate, 0.14 M sodium chloride, 0.01 M potassium chloride, Pierce, Rockford, IL with 0.05% Tween 20 (Sigma)), a 1.0 mg/mL solution of Bovine Serum Albumin (BSA, Fisher, Pittsburgh, PA) in PBST, or PBST only (the control). The volume used to hydrate the meal pad was adjusted in order to obtain a final concentration in the diet of 0.8 mg of anti-BSA or BSA/g wet diet. Fourth stadium, day 0 *H. virescens* larvae as described earlier were transferred to the hydrated meal pads (one larva per cup), and the cups sealed with a paper lid. All assays were conducted under standard rearing conditions as described previously. The larvae were observed after 30 min, and those that did not begin

feeding were removed from the test. Larvae were observed again after 8 h, and those that had not consumed most of the meal pad were discarded. At this time, an additional 80-120 mg meal pad was added to each rearing cup for the 16 h bioassay. After 16 h, larvae that had not consumed most of their second meal pad were discarded.

At the end of the feeding assay (at 8 or 16 h) larvae were removed from the diet. Any diet or feces on larvae was removed with a camel hair brush. Each larva was then rinsed with a stream of 2-3 mL of PBST and blotted dry on a Kimwipe (Kimberly-Clark, Roswell, GA). Hemolymph was collected from a cut thoracic leg using scissors and transferred as a small drop to the surface of Para-film (Para-film, Menasha, WI). The scissors were rinsed with PBST and dried with a Kimwipe between larvae to prevent cross-contamination. Using a 10 μL pipettman (Rainin, Emeryville, CA), 5.0 μL of the hemolymph from the Para-film was aliquoted into 495 μL of cold PBST (4^{o} C) in a 2.5 mL microcentrifuge tube and immediately vortexed. All samples were stored at -80°C until assayed. Freeze/thawing had no affect on assay results.

In order to verify that the external washes were adequate in removing all of the diet contaminants from the cuticle, budworms after the 8 h incubation were subjected to an additional 300 μL stream of PBST. The wash was collected and stored at -80°C until needed for assay.

Stability of anti-BSA and BSA in Artificial Diet

Meal pads hydrated with BSA, anti-BSA, or PBST were incubated for 0 and 24 h under the same conditions as described for the feeding bioassay. After incubation, the diet was homogenized in PBST for 15 sec (4^{o} C) at full speed with a polytron PT10/35

homogenizer with PTA 10 generator (Brinkmann, Westbury, NY). The generator probe was rinsed at full speed in excess PBST between samples to prevent cross-contamination. A 500 μL aliquot of the homogenate was centrifuged at 960 X g (4^{o} C) for 2 min, and then 50 μL of the supernatant added to 450 μL of ice cold PBST for assay.

Topical Bioassay

Five microliters of a 1.0 mg/mL solution of anti-BSA or BSA in DMSO or 5μL of DMSO only (control) was topically applied using a 10 μL pipettman to the dorsum of the abdomen of fourth stadium, day 0 budworms. Treated larvae were then transferred to a 1-oz cup (1 larva/cup) without food. The larvae were incubated at standard rearing conditions. After 4 h, the external surface of the larvae was cleaned, and hemolymph samples collected as described earlier. All samples were stored at -80°C until assayed.

Injection of Anti-BSA and BSA into the Hemocoel

One microliter of a 20 mg/mL solution of anti-BSA in PBST, of a 20 mg/mL solution of BSA in PBST or of PBST alone (control) was injected into fourth stadium, d 0 *H. virescens*. The injections were made dorsolaterally 2 or 3 segments from the posterior using a repeating dispenser fitted with a 10 μL glass syringe with a 33-gauge needle (Hamilton, Reno, NV). The needle was inserted into the larvae just under internal to the epidermis with the end of the needle extending at least 2 segments anterior to the insertion site. After injection, the needle was left in place for 5 sec. After the injection the needle was removed, and any larvae that demonstrated bleeding were discarded. Injected larvae were placed individually into 1-oz diet cups without food and incubated at 25°C.

Hemolymph was collected 5, 15, 30, 60 and 120 min after injection and samples stored at -80°C until assayed.

Incubations of Anti-BSA and BSA in Plasma

Hemolymph (1.5 mL) was collected from multiple fourth stadium, d 0 larvae (5-10 μL per larva) as described earlier and pooled in a 2.0 mL microcentrifuge tube containing 5 mg phenylthiourea (PTU, Sigma-Aldrich, Saint Louis, MO) on ice. The tube was shaken repeatedly during hemolymph collection to mix the PTU, and then after the collection was completed the tube was centrifuged at 1000 X g for 5 min at 25° C. Aliquots (19.8 μL) of the supernatant (plasma) at 25° C were added in separate 0.2 mL microcentrifuge tubes to 0.2 μL of a 20mg/mL solution of anti-BSA in PBST, 0.2 μL of a 20 mg/mL solution of BSA in PBST, or 0.2 μL of PBST alone (control) and incubated for 5, 15, 30, 60 or 120 min at the same temperature. After incubation, 5 μL of the hemolymph from each sample was added to 495 μL of PBST (4^{o} C). All samples were stored at -80°C until assayed.

Fecal Analysis

Twelve h after feeding on meal pads containing anti-BSA, BSA, or PBST alone (control), larvae were removed from their rearing cups and washed as described earlier. The larvae were transferred to a new cup without diet and returned to standard rearing conditions. After 4 h, the feces was collected and frozen at –80° for 24 h followed by lyophilization for 12 h (same conditions as described earlier). The feces was then

weighed and homogenized as described before for meal pads. The homogenate was transferred to a microcentrifuge tube and centrifuged for 2 min at 960 X g (4^{o} C). The supernatant was stored at -80^{o} C until assayed.

Enzyme Linked Immunosorbent Assay (ELISA)

Detection of anti-BSA antibody

Wells of a 96-well microtiter plate (Falcon, Franklin Lakes, NJ) were coated with BSA to serve as the immobile phase of the ELISA. The BSA was dissolved in a coating buffer (pH 9.4, 0.2 M sodium carbonate bicarbonate, Pierce) to make a 500 ng/mL solution. Aliquots (50 μL) of the BSA solution were applied to each microtiter plate well and incubated for 15 h at 5° C. The plates were emptied and washed 3 times with PBST. Samples (50 μL) to be assayed were added to the wells of the microtiter plate. The plate was covered and incubated 3 h at 25° C before being emptied and rinsed 3 times with PBST. Alkaline phosphatase labeled, goat anti-rabbit antiserum (Sigma) diluted 1:2000 in PBST was added to each well (50 μL). The plates were incubated 3 h at 25° C before being emptied and rinsed 3 times with PBST. P-nitrophenyl phosphate substrate solution (PNPP, 50 μL) was added to all wells. The PNPP solution was made by adding 1 PNPP disodium salt tablet to 8 mL water and 2 mL 5x Diethanolamine substrate buffer according to the manufacturer's instructions (Pierce). The plate was covered and incubated on a rocker shaker for 30 min at 25° C. The optical Absorbance was read at 405 nm on a MDC PR microplate reader using SOFTmax PRO version 3.1.2 (Molecular Devices Corporation, Sunnyvale, CA). Results were compared to a standard curve generated using known concentrations of anti-BSA in PBST.

Detection of BSA

Wells of 96-well microtiter plates were coated with mouse (monoclonal) anti-BSA antiserum (Sigma) to serve as the immobile phase of the ELISA. The anti-BSA was dissolved in coating buffer to make a 500 ng/mL solution. Aliquots (50 μL) of the anti-BSA solution were applied to each microtiter plate well and incubated for 15 h at 5° C. The plates were emptied and washed 3 times with PBST. Samples (50 μL) for assay were added to the wells of the microtiter plate. The plate was covered and incubated 3 h at 25° C before being emptied and rinsed 3 times with PBST. Aliquots (50 μL) of a rabbit anti-BSA dilution in PBST (1:2000) were applied to each microtiter plate well and incubated for 3 h at 25° C before being emptied and rinsed 3 times with PBST. Alkaline phosphatase labeled, goat anti-rabbit antiserum diluted 1:2000 in PBST was added to each well (50 μL). The plates were incubated 3 h at 25° C before being emptied and rinsed 3 times with PBST. PNPP solution (50 μL) was added to all wells. The plate was covered and incubated on a rocker shaker for 30 min at 25° C. The optical Absorbance was read at 405 nm on a MDC plate reader. Results were compared to a standard curve generated by using BSA in PBST.

Detection of Anti-BSA and BSA by Western Blot

Detection of BSA by Western Blot under non-denaturing conditions

A Zaxis (Hudson, OH) System 2000 EP was filled with a Tris Borate EDTA buffer buffer (TBE buffer, 0.045 M Tris-borate (Fisher) and 0.001 M EDTA (Fisher)). Five μL of each sample (containing 0.1 μg of BSA) was mixed with 5 μL of loading buffer (0.2%

bromophenol blue (Fisher) and 10% sucrose (Fisher)) and separated by electrophoresis on a 10x10 cm 4-12% acrylamide Tris Borate EDTA (TBE) gel with a 4% stacking layer (Zaxis). The gel was run at 150 V until the dye markers were approximately 1 cm from the base of the gel.

The proteins were transferred by electroblotting to an Immobilon P Transfer Membrane (Millipore Corporation, Bedford, MA), pore size 0.45μm. The membrane was soaked in ethanol before being placed on the gel. The transfer was done using the Biorad (Richmond, CA) Electro-Eluter Model 442 system with a transfer buffer (39mM glycine (Fisher), 48mM Tris base (Fisher), 0.037% SDS (Fisher), and 20% methanol (Fisher)). The transfer was run for 2 h at 25° C at 50 mA.

After the transfer the membrane was incubated in 50 mL of a 1:1000 dilution of rabbit (polyclonal) anti-BSA in PBST for 2 h on a rocker shaker at 25° C. The anti-BSA solution was removed, and the membrane was washed 3 times with 50 mL PBST. Next, the membrane was incubated with 50 mL of a 1:1000 dilution of alkaline phosphatase labeled anti-rabbit (Sigma) in PBST for 2 h on the rocker shaker at 25° C. The membrane was washed three times with PBST. Finally 2 mL of NBT/BCIP solution (nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate, Sigma) was added over the surface of the membrane. The color was allowed to develop for 10 min, and then the membrane was scanned.

Detection of anti-BSA and BSA under denaturing conditions

Five μL of each sample (containing 0.1 μg anti-BSA or BSA) was mixed with 5 μL of 2x Tris HCl SDS Sample Prep Buffer containing 2-mercaptoethanol (Zaxis). Samples were heated to 90° C in a heated block for 3-5 min. A Zaxis System 2000 EP was filled

with 1x Tris-Glycine Running Buffer (Tris 0.025 M, Glycine 0.192 M and SDS 0.1%). Proteins were separated by electrophoresis on a 10x10 cm 4-20% polyacrylamide Tris-Glycine Gel with a 4% stacking layer (Zaxis). The gel was run at 150 V until the dye markers were approximately 1 cm from the base of the gel. Proteins were transferred to a nylon membrane, and detected as described earlier.

RESULTS AND DISCUSSION

Stability of Anti-BSA and BSA in Meal Pads

A critical aspect of our feeding bioassay to examine protein movement across the insect digestive system is to accurately incorporate test proteins into artificial insect diet. In addition, once the proteins are in the diet, it is important that they are not subjected to degradation or any changes that might affect their function or immunoreactivity during the course of our assay. The technique of using dehydrated meal pads for monitoring Lepidopteran insect resistance to pesticides was recently developed by Roe et al. (2000, 2003) and Bailey et al. (2001). The dehydrated meal pad matrix is constructed so protein toxins like Bt Cry1Ac could be added in water to the dried diet, producing an instant hydrated insect meal. In the case of resistance diagnosis, the dehydrated insect meal pad was formulated to also include an indicator dye. Insects that were resistant to a diagnostic dose of Bt Cry1Ac were able to feed on the diet containing the protein toxin, and resistance could be detected simply by observing for the presence of blue fecal pellets. Susceptible budworms did not produce blue feces.

There are a number of advantages of using these dehydrated meal pads for our feeding bioassays. The addition of anti-BSA and BSA to the diet matrix results in a

uniform dispersal of the test protein in the diet (Bailey et al., 1998). Since this addition is conducted at room temperature, we can accurately and with good precision produce meal pads containing known concentrations of BSA and anti-BSA. Bailey et al. (1998) also found that proteins like Cry1Ac were not degraded during the course of resistance bioassays and that insect growth and development on hydrated meal pads were essentially identical to that of standard artificial diet.

It was critical for our current studies to determine the stability of anti-BSA and BSA in this system. To examine this question, dehydrated meal pads were hydrated with solutions of anti-BSA or BSA, the hydrated meal pads incubated under standard insect rearing conditions, and the concentrations of anti-BSA and BSA determined after 24 h by ELISA. The indirect ELISA method (Voller, 1979) was used to detect anti-BSA while a modified version of the double antibody sandwich ELISA (Voller, 1979) was used to detect BSA. The lower limit of detection for both methods was 80 ng of protein/g-diet. The concentration of anti-BSA detected in the anti-BSA diet was 497.4 ± 13.4 μg/g diet (average ± 1 SEM) at 0 h and 496.6 ± 15.1 μg/g diet at 24 h (Fig. 1a). The concentration of BSA extracted from the BSA diet was 489.6 ± 21.8 μg/g diet at 0 h and 486.0 ± 13.9 μg/g diet at 24 h (Fig. 1b). Since the concentrations were not significantly different between 0 and 24 h for both proteins (Fig. 1; t-test, α=0.05, p=0.49 for anti-BSA and p=0.45 for BSA), it is apparent that the protein concentration for BSA and anti-BSA using dehydrated meal pads was constant during the course of our feeding bioassays. It is also important to note that there were no non-specific, immunoreactive substances in the meal pads that might interfere with the detection of BSA or anti-BSA. The recovery of anti-BSA and BSA at 0 h was 62.1% and 61.2%, respectively, and this was unchanged at

24 h for both proteins. The loss in detectable anti-BSA and BSA was the result of an incomplete extraction of these proteins from the diet solids, which were removed during homogenate clarification prior to the ELISA analysis.

Although by ELISA there was no change in BSA or anti-BSA protein concentration in meal pads, protein degradation is not completely excluded by this analysis. To further examine this question, Western blots were conducted under native and denaturing conditions. Fig. 2 shows the electrophoretic mobility under native conditions of BSA in the hydration solution (lane 1) and at 0 (lane 2) and 24 h (lane 3) after the addition of the BSA to meal pads. The BSA monomer with a molecular weight of 66k, the dimer (132k), and the trimer (198k) were found in all three samples with the relative proportion of each approximately the same in lanes 1-3. These results suggested that not only was there no change in concentration of BSA in meal pads as determined by ELISA, but there was also no reduction in the protein molecular weight, no change in the multimeric structure of the protein, or the relative abundance of each multimeric form. Western blots under denaturing conditions for these same samples (Fig 3, lanes 1-3) further confirmed no degradation of the BSA in meal pads. Note also that band width for BSA from the diet samples were equivalent to that from the hydration solution, further indicating no BSA degradation in our meal pads over a 24 h period.

Anti-BSA could not be effectively resolved on native gels because of its large molecular weight and poor electrophoretic mobility. The antibody is tetrametric in structure (150 kDa in molecular weight; 2 heavy chains and 2 light chains). On Western blots using denaturing gels, we were able to resolve both the heavy (50 kDa) and light (25 kDa) chains (Fig. 4). No changes in molecular weight were noted between the anti-

BSA in the hydration solution (Fig. 4, lane 1) and that for the hydrated meal pads at 0 and 24 h (lanes 2 and 3, respectively). The proportion of light versus heavy bands was approximately the same as indicated by band intensities for all three samples. It was clear from both the ELISA and Western Blot analyses that BSA and anti-BSA were unchanged in hydrated meal pads for up to 24 h.

Protein Movement Across the Insect Digestive System

Newly molted, Day 0-fourth stadium tobacco budworm, *H. virescens*, larvae were used in our feeding bioassays to determine protein movement across the insect digestive system. Larvae, 45–55 mg in weight, were removed from diet and placed individually in rearing cups without diet for 6 h under standard rearing conditions. Only larvae that molted to the fourth stadium during this 6 h period were used for bioassay. These larvae were then transferred individually to rearing cups containing a single hydrated meal pad per cup. The larvae started feeding in 5-30 min and were continuously observed during this time period to determine the exact start time of feeding (0 h). Larvae were at 0 h when they were found in contact with the meal pad and were exhibiting obvious feeding behavior. Insects that were continuously feeding for the next 8 h consumed approximately one-third of their meal pads. Larvae not exhibiting this behavior at 8 h were discarded. To ensure continuous feeding between 8 and 16 h, the old diet was removed at 8 h and a new meal pad added to the rearing cup for each larva. At 16 h, only those larvae that consumed approximately one-third of the second meal pad were used for further testing. Larvae in our studies produce feces from their meal in no more than 6 h. Therefore, using this methodology we were confident that the digestive system at the

time of sampling (8 and 16 h) contained food from meal pads that had been hydrated with BSA or anti-BSA protein. Recall from our earlier discussion, no degradation of BSA or anti-BSA was noted by ELISA and Western blots in hydrated meal pads after 24 h.

After feeding on the diet containing anti-BSA or BSA, feces from *H. virescens* was analyzed to determine whether either protein could survive digestion. Larvae selected and bioassayed as previously described were allowed to feed on meal pads containing either 0.8 mg of anti-BSA or BSA/g-diet for 12 h under standard insect rearing conditions. Larvae were then transferred to a rearing cup without diet for 4 h, and the feces collected and assayed by ELISA for BSA and anti-BSA. Neither protein was detected. The lower limit of detection for these assays was 100 ng/g-feces. These results indicated two possible pathways for the elimination of BSA and anti-BSA from the diet as the food moves through the budworm digestive system: (i) absorption and/or (ii) degradation to a non-functional anti-BSA protein and a non-immunoreactive form of BSA.

To address the question of whether anti-BSA and/or BSA could be absorbed intact from the diet, hemolymph samples were taken at 8 and 16 h after feeding on meal pads containing 0.8 mg of anti-BSA or BSA/g-diet, respectively. The concentration of anti-BSA in larval hemolymph after feeding for 8 and 16 h on meal pads containing anti-BSA was 2430±125 (average ±1 SEM) and 3459±105 ng/mL, respectively (Fig. 5A). No anti-BSA was detected in hemolymph at 0 h or in hemolymph of larvae that fed for 8 and 16 h on meal pads containing no anti-BSA. The lower limit of detection for anti-BSA in hemolymph in these experiments was 1 ng/mL. The concentration of anti-BSA in the hemolymph at 0 h was significantly different from that at 8 and 16 h (t-test, α=0.05, p=3.89E-39 for 8 h, p=3.26E-62 for 16 h).

The BSA concentration in larval hemolymph at 8 and 16 h was 1547±132 and 1623±122 ng/mL, respectively (Fig. 5B). No BSA was detected in hemolymph at 0 h or in hemolymph of larvae that fed for 8 and 16 h on meal pads without BSA. The lower limit of detection for BSA in hemolymph in these experiments was 1 ng/mL. The concentration of BSA in the hemolymph at 0 h was significantly different from that at 8 and 16 h (t-test, α=0.05, p=1.20E-19 for 8 h, p=1.11E-22 for 16 h).

From these studies, it is apparent that anti-BSA and BSA as detected by ELISA can survive to some degree the digestive processes that occur in the insect gut and can move from the diet into the hemolymph of fourth stadium budworms. Ben-Yakir (1996) also found that anti-ovalbumin could survive digestion and enter the hemolymph of the European corn borer, *Ostrinia* nubilalis. At similar diet concentrations as used in our studies with the budworm, the ratio of anti-ovalbumin in the hemolymph as compared to the diet was 1:500 (wt:wt). The ratios for anti-BSA in the budworm at 8 and 16 h were 1:329 and 1:231, respectively, and for BSA was 1:517 and 1:491, respectively.

Even though the protein concentration in the diet on a wt:wt basis was the same for both BSA and anti-BSA, the anti-BSA concentration in hemolymph exceeded that for BSA at both 8 (t-test, α=0.05, p=2.32E-06) and 16 h (t-test, α=0.05, p=1.38E-19). Interestingly, the anti-BSA concentration was higher than the concentration for BSA in the hemolymph despite the fact that anti-BSA has a higher molecular weight (150 kDa) as compared to the BSA monomer (66 kDa) and that the molar concentration of BSA in the diet (12 nmol) was about twice that for anti-BSA (5 nmol). These results indicate that hemolymph accumulation of these proteins is not simply a result of diet concentration and that different protein species may accumulate in the hemolymph at different rates

despite large differences in their molecular weights. The mechanisms for these different accumulation rates are unknown but could include differing rates of degradation or sequestration in the gut lumen, transport across the digestive system epithelium, and degradation and sequestration in the hemocoel. Structure activity and pharmocodynamic studies are needed to better understand the importance of protein structure on the mechanism(s) responsible for hemolymph accumulation. This is an under studied area of insect physiology.

Western blots were used to determine whether any degradation occurred to anti-BSA or BSA once the proteins had moved into the hemolymph. The BSA hydration solution and a hemolymph sample from *H. virescens* after 8 and 16 h of feeding were analyzed by native gel electrophoresis and Western blotting. All three multimeric forms of BSA were found in the budworm hemolymph at the same relative intensities as in the BSA solution used to make the protein diet (Fig. 2; compare lanes 1, 4 and 5). These results suggest that BSA is passing through the gut intact including even maintaining its multimeric structure and/or the protein is degraded to the monomer (or dimer) and then regains its native multimeric form once in the hemolymph. However, the abundance of each BSA form was identical in the hydration solution (Fig. 2, lane 1), diet (Fig. 2, lane 2) and hemolymph (lanes 4 and 5), suggesting that the multimeric structure of BSA was unchanged as it moved from the diet to the hemolymph. The Western blot analysis under denaturing conditions (Fig. 3, compare lanes 1 and 2 with lanes 4 and 5) further shows that no degradation products of BSA was detected in the hemolymph after feeding on BSA-meal pads for 8 and 16 h. In addition, no degradation products for anti-BSA were found in hemolymph after 8 and 16 h of feeding (Fig. 4, compare lanes 1 and 2 with lanes

4 and 5). These results argue that if any degradation occurs in the hemolymph, the degradation must be rapid and complete to the point that the products lose their immunoreactivity to the BSA polyclonal antibody. Alternatively, no degradation may be occurring or the proteins may be sequestered from the hemolymph. Further discussion of this will be presented later.

Routes of Movement of Anti-BSA and BSA into Hemolymph during Feeding

In the feeding bioassays just discussed to examine the movement of proteins across the insect digestive system, budworm larvae often crawled and for extended periods during the bioassay rested on the diet surface. Under these conditions, the insect cuticle is in contact with the diet and anti-BSA or BSA could be transferred to the cuticle surface. To limit this transfer as much as possible, we used insect meal pads with a relatively small surface area as compared to that of the inside of the rearing cup. The insects under these test conditions sometimes remained on the container surface while feeding but cuticle contamination could not be eliminated. To prevent any contamination of hemolymph with anti-BSA or BSA from the cuticle during bleeding, the larvae were cleaned with a camel hair brushed and rinsed with 2-3 mL of PBST. After this cleaning, we could not detect anti-BSA or BSA from an additional 300 μL rinse. The lower limit of detection by ELISA was 1 ng/mL rinse.

Another possible route of movement of anti-BSA and BSA into the hemolymph in our feeding bioassay was movement across the insect cuticle. To examine this question, 5 μg of anti-BSA or BSA was topically applied in 5 μL of DMSO to the dorsum of the abdomen of fourth stadium, day 0 tobacco budworm larvae. The larvae were incubated

under standard rearing conditions for 4 h, the cuticle cleaned of the applied protein and a hemolymph sample analyzed by ELISA. No anti-BSA or BSA was detected in the hemolymph. The lower limited of detection was 1 ng/mL hemolymph. It is clear from these results that the only possible route of entry of anti-BSA and BSA in our feeding bioassays was the movement of these proteins intact across the insect gut. In the case of BSA, the multimeric structure of the protein and in the case of anti-BSA its antigen affinity was maintained during this transfer.

The transfer of intact proteins across the digestive system and into the circulatory system of mammals was documented as early as 1936 by Verzár and McDougall. Macromolecular (including protein) transport across the mammalian digestive system has been discussed in the following reviews: Silk (1980), Udall and Walker (1982), Silk and Keohane (1983), Gardner (1984, 1988), Weiner (1988), and Pácha (2000). The transport of macromolecules across the stomach and small intestinal wall is important during early postnatal life because it facilitates the absorption of growth factors, antigens, and immunoglobulin from maternal colostrum and milk. In early mammalian development the transport of macromolecules occurs in enterocytes and follows two pathways: specific receptor-mediated transcytosis and nonspecific transcytosis (Pácha, 2000). In specific receptor-mediated transepithelial transport of immunoglobulins the IgG present in milk (or colostrum) bind to a specific receptor in the apical membrane, the complex is endocytosed, moved through the cell within a transport vesicle, and secreted into the contraluminal compartment. In some mammals, it possible to observe a massive absorption of IgG via a nonselective endocytotic pathway when intraluminal macromolecules are endocytosed, partially destroyed, and partially transported across the

enterocytes. The high permeability of the intestinal epithelium for macromolecules declines after birth in a process called gut closure (Pácha, 2000). However the mature mammalian small intestine retains the ability to absorb macromolecules to a small extent (Weiner, 1988). The mechanisms by which proteins can cross the mature mammalian small intestine are not as well studied as in immature mammals, but a few studies have been conducted to understand this process. Danforth and Moore (1959) were able to cause hypoglycemia in the adult rat by injecting insulin (with protease inhibitors) into isolated loops of small intestine. Bockman and Winborn (1966), using electron microscopy, visualized the presence of ferritin in membrane bound vesicles of adult hamster intestinal epithelial cells and lamina propria following intraluminal injections. Using electron microscopy and cytochemical techniques, Walker et al. (1983) found small quantities of horseradish peroxidase (HRP) were transported into the intestinal epithelium by an endocytotic mechanism and subsequently released into the intercellular space below the tight junction in adult rats. Using 2 small proteins, microperoxidase (1900 Da) and Cytochrome C (12,300 Da), Madara and Trier (1982) found that neither protein was able to penetrate the tight junctions between rat ileum epithelial cells.

In comparison to mammals, very little is known about the movement of proteins across the insect gut. Modespacher et al. (1986) showed HRP moves into the intracellular clefts of the anterior-part and middle-part (0.5 and 1 h after feeding) of the midgut in the tsetse fly, *Glossina moritans*. One h after feeding HRP was present in basal labyrinth of the midgut epithelium in the anterior and middle-parts. After 2 h most basal parts of the basal labyrinth were preferentially labeled as well as the basal lamina, indicating HRP reached the hemolymph. No HRP was found in the posterior-part of the midgut, and

Modespacher found no indication that pinocytosis occurs in the cells. Fishman and Zlotkin (1984) showed that HRP enters the epithelial gut cells of the flesh fly, *Sarcophaga faculata*, through the microvilli 30 min after feeding. Next HRP diffuses across the cytoplasm of the cell and into the hemolymph across the gut wall via the inner plasma membrane, basal membrane, and the outer layer of muscles. The continuous diffusion throughout the cytoplasm suggests that pinocytotic uptake or vesicular transport, common in mammalian systems, is unlikely. Using immunoenzyme labeling, Hatfield (1988) found mouse IgG bound to the midgut epithelium of *Aedes aegypti* after feeding. Next using immunogold labeling of thin sections Hatfield detected mouse IgG in the cytoplasm of the midgut epithelium microvilli. Finally, mouse IgG was found in the mosquito hemolymph using ELISA. The IgG was not bound to any other tissue outside of the gut. Hatfield proposed that the IgG might actively and selectively move through the midgut epithelium as described earlier in the mammalian discussion. Also he proposed a passive transfer of IgG between the epithelial cells, a "leaky" midgut, which is the explanation for the rapid appearance of arboviruses in a bloodmeal after feeding (Boorman, 1960; Miles et al., 1973; Hardy et al., 1983). The mechanisms by which proteins enter the hemolymph of non-hematophagous insects have never been studied.

Fate of Anti-BSA and BSA in Hemolymph

We discussed earlier that the BSA concentration in the hemolymph of fourth stadium budworms feeding on meal pads containing 0.8 mg anti-BSA/g-diet increased from 0 (at 0h) to greater than 1,500 ng/mL after 8 h (Fig. 5b). However, there was no change in concentration from 8 to 16 h (t-test, α=0.05, p=0.335), even though the insects were

continuing to feed and consumed about the same amount of diet from 0 to 8 h and 8 to 16 h. For anti-BSA there was a significant increase in concentration between 8 to 16 h (t-test, α=0.05, p=2.55E-09), a 35% increase. These results suggested that either the amount of hemolymph uptake of BSA and anti-BSA (to a lesser extent) was limited or that the protein was being degraded or removed from the hemolymph during the course of the assay. Wigglesworth (1943) found that hemoglobin when injected into the hemolymph of *Rhodinus prolixus* was denatured and oxidized to kathemoglobin, which was then sequestered by the ovaries, salivary glands and pericardial cells (Wigglesworth, 1943). Ichinose et al. (1992) and Bonning et al. (1996) found that juvenile hormone esterase in the hemolymph of the larval tobacco budworm, *Manduca sexta*, was sequestered in the pericardial cells by receptor-mediated endocytosis. The enzyme was eventually degraded in the lysosomes.

Twenty μg of anti-BSA or BSA was injected into the hemocoel of day 0, fourth stadium budworms to determine the turnover rate of these proteins. Hemolymph samples were collected at different times after injected and the anti-BSA and BSA concentration analyzed by ELISA. The concentration of anti-BSA and BSA decreased to 0.6 and 20.0%, respectively, of their original concentration 120 min after the injection (Fig. 6). The concentration of anti-BSA and BSA in the hemolymph 5 minutes after injection was 132±4.0 and 126±6.0 (average ± 1 SEM) μg protein/mL hemolymph.

Similar studies were conducted with whole plasma held *in vitro*. The concentration of anti-BSA and BSA at 5 minutes after addition of protein to pooled plasma was 204±10.8 and 193±2.5 (average ± 1 SEM) μg protein/mL hemolymph in these experiments, similar to the concentration used *in vivo*. The concentration of anti-BSA and

BSA decreased to 81.5 and 57.5%, respectively, of the original concentration 120 min after the injection (Fig. 6). The rate of degradation of anti-BSA and BSA was greater *in vivo* than *in vitro* (weighted linear regression analysis, $\alpha=0.05$, $p=0.0004$ for anti-BSA and $p<0.0001$ for BSA), suggesting that sequestration in addition to hemolymph degradation is a factor in the turnover of these proteins in budworm hemolymph. Sequestration of proteins from insect hemolymph has been demonstrated in other insect species as previously discussed. In addition, these results suggest that the level of accumulation of anti-BSA and BSA in hemolymph from meal pads must be affected by a balance between uptake from the digestive system and the likely turnover of these proteins in the hemolymph. It is also worth noting that both in our *in vivo* and *in vitro* experiments, the turnover rate for BSA was greater than that for anti-BSA (weighted linear regression analysis, $\alpha=0.05$, $p=0.0157$ for *in vivo*, $p=0.0089$ for *in vitro*).

In summary, tobacco budworms feeding on artificial diet containing either anti-BSA or BSA were able to transfer both proteins intact across the gut epithelium. BSA retained its multimeric structure in hemolymph as determined by Western blots, and anti-BSA maintained its affinity for BSA as determined by ELISA. Degradation of both proteins was not noted in hemolymph at different times after the beginning of feeding. Neither anti-BSA nor BSA will cross the outside, insect cuticle. The level of accumulation of these two proteins in budworm hemolymph is the result of at least two processes—the rate of transfer from the insect diet and the rate of their turnover in the hemolymph. Turnover in the hemolymph appears to be the result of degradation and sequestration, although more detailed studies are needed to understand these mechanisms.

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FIGURES AND FIGURE CAPTIONS

Fig. 1. Stability of anti-BSA and BSA in lyophilized diet. Lyophilized insect diet was reconstituted with solutions of anti-BSA or BSA (final concentration in diet was 0.8 mg BSA or anti-BSA/ g diet). At 0 and 24 h the diet was homogenized and proteins were extracted. Samples were analyzed for the presence of anti-BSA or BSA by ELISA. Results of 5 homogenations per time point were averaged and presented as the average +/- 1 Standard Error of the Mean. (ND = not detected)

Fig. 2. Determination of structure of BSA by Western Blot under non-denaturing conditions. BSA samples were run on a 4-12% acrylamide tris-borate EDTA gradient gel and transferred by electroblotting to a nylon membrane. (samples contained 0.1 μg of BSA in 10 μL) 1, solution of BSA used to make protein diet; 2, BSA from diet at 0 h; 3, BSA from diet at 24 h; 4, hemolymph sample from *H. virescens* after feeding 8 h on BSA diet; 5, hemolymph sample from *H. virescens* after feeding 16 h on BSA diet.

Fig. 3. Determination of structure of BSA by Western Blot under denaturing conditions. BSA samples were run on a 4-20% polyacrylamide tris-glycine gradient gel and transferred by electroblotting to a nylon membrane. (samples contained 0.1 μg of BSA in 10 μL) 1, solution of BSA used to make protein diet; 2, BSA from diet at 0 h; 3, BSA from diet at 24 h; 4, hemolymph sample from *H. virescens* after feeding 8 h on BSA diet; 5, hemolymph sample from *H. virescens* after feeding 16 h on BSA diet.

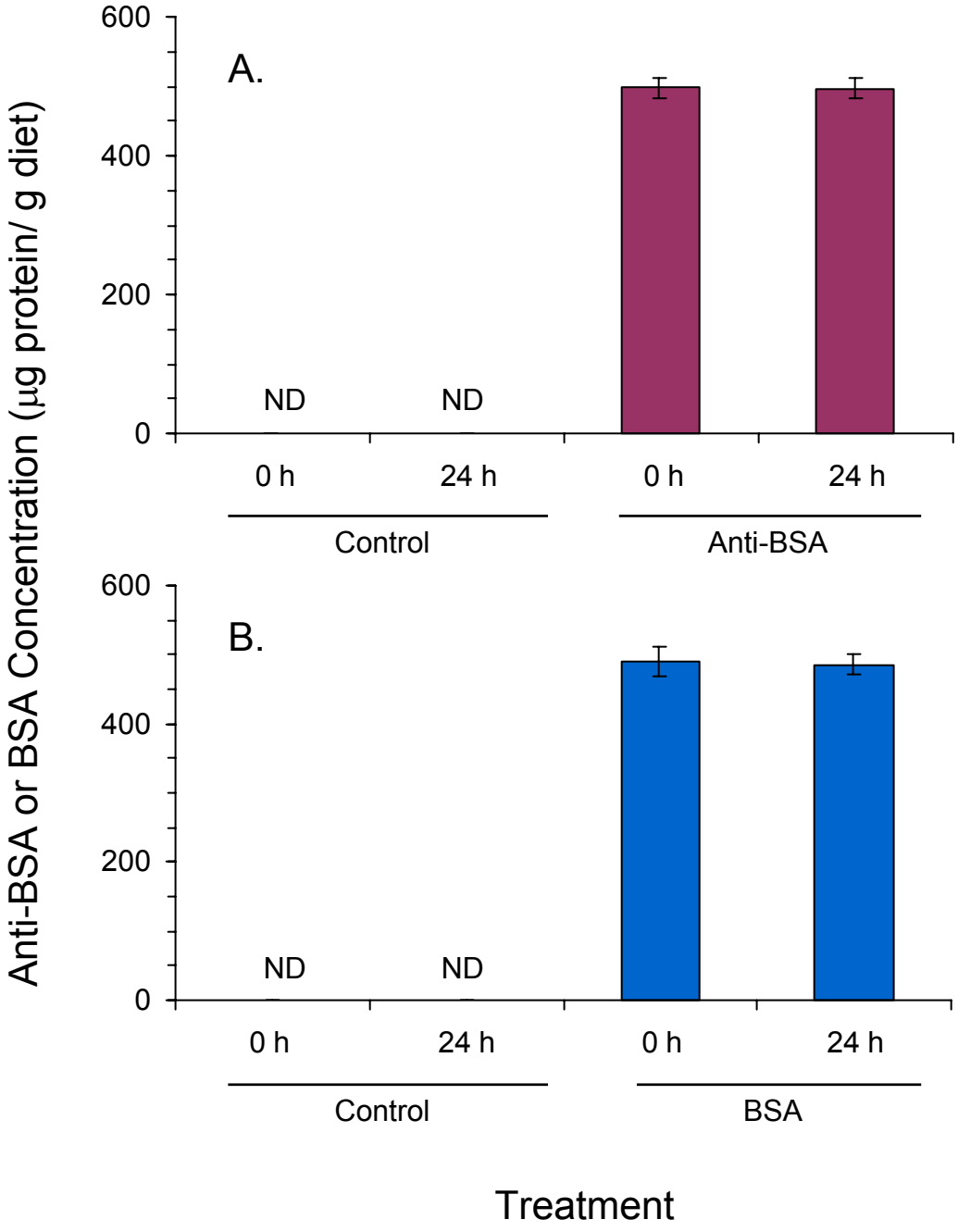
Fig. 4. Determination of structure of anti-BSA by Western Blot under denaturing conditions. Anti-BSA samples were run on a 4-20% polyacrylamide tris-glycine gradient gel and transferred by electroblotting to nylon membrane. (samples contained 0.1 μg of anti-BSA in 10 μL) 1, solution of anti-BSA used to make protein diet; 2, anti-BSA from diet at 0 h; 3, anti-BSA from diet at 24 h; 4, hemolymph sample from *H. virescens* after feeding 8 h on anti-BSA diet; 5, hemolymph sample from *H. virescens* after feeding 16 h on anti-BSA diet.

Fig. 5. Passage of anti-BSA and BSA across the digestive system into hemolymph after feeding. Fourth Stadium, Day 0 *H. virescens* larvae were fed on diet containing anti-BSA or BSA for 8, or 16 h. (concentration in diet 0.8 mg BSA or anti-BSA/ g diet) Hemolymph was collected and analyzed for the presence of anti-BSA or BSA by ELISA. Results of feeding assay were averaged and presented as the average +/- 1 Standard Error of the Mean. (ND = not detected)

Fig. 6. *In vivo* injections of anti-BSA and BSA. Twenty μg of anti-BSA or BSA in PBST was injected into 4th Stadium, Day 0 *H. virescens* larvae. Hemolymph was collected and analyzed for the presence of anti-BSA or BSA by ELISA at 5, 15, 30, 60, or 120 min. Results of 10 injections per time point were averaged and presented as the average +/- 1 Standard Error of the Mean.

Fig. 7. *In vitro* stability of anti-BSA and BSA in plasma. Twenty μg of anti-BSA or BSA in PBST was added to pooled plasma from 4th Stadium, Day 0 *H. virescens* larvae.

Plasma was incubated for 5, 15, 30, 60, or 120 min at room temperature and analyzed for the presence of anti-BSA or BSA by ELISA. Results of 10 incubations per time point were averaged and presented as the average +/- 1 Standard Error of the Mean.


A.
B.
600
400
200
0
ND
ND
0 h
24 h
0 h
24 h
Control
Anti-BSA
BSA
Anti-BSA or BSA Concentration (μg protein/ g diet)
Treatment

Trimer 198 kDa

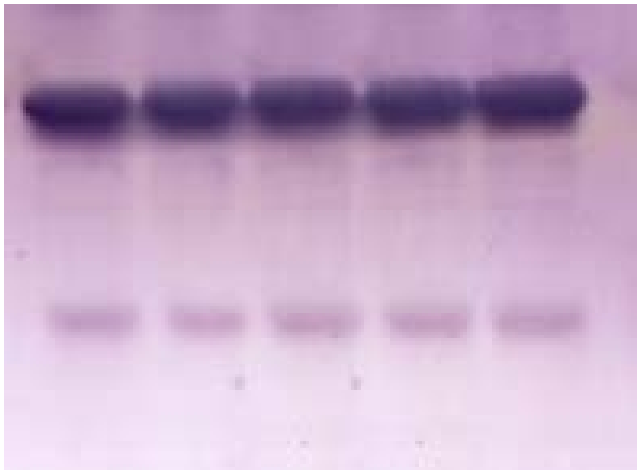
Dimer 132 kDa

Monomer 66 kDa

1 2 3 4 5

Monomer 66 kDa

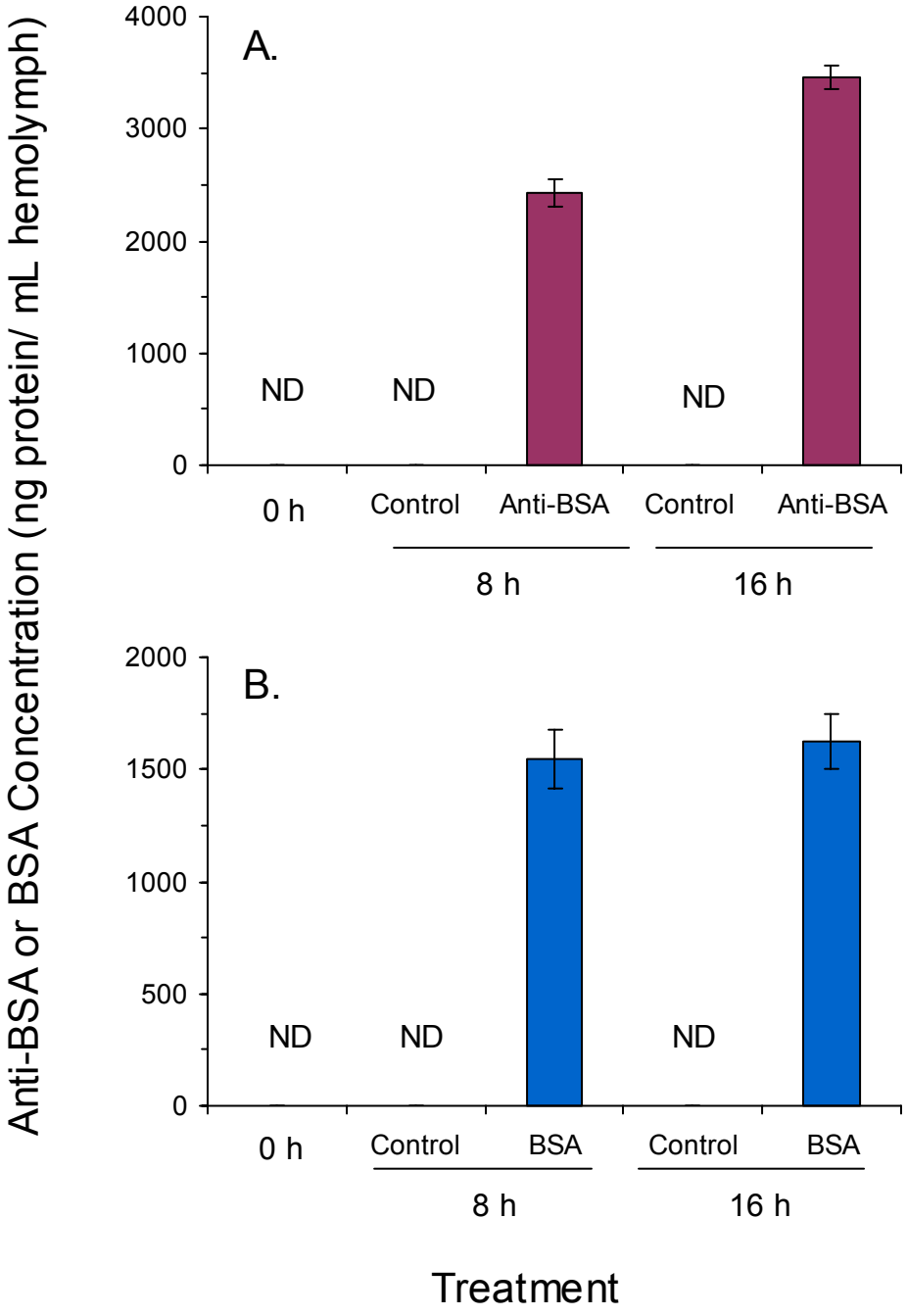
1 2 3 4 5

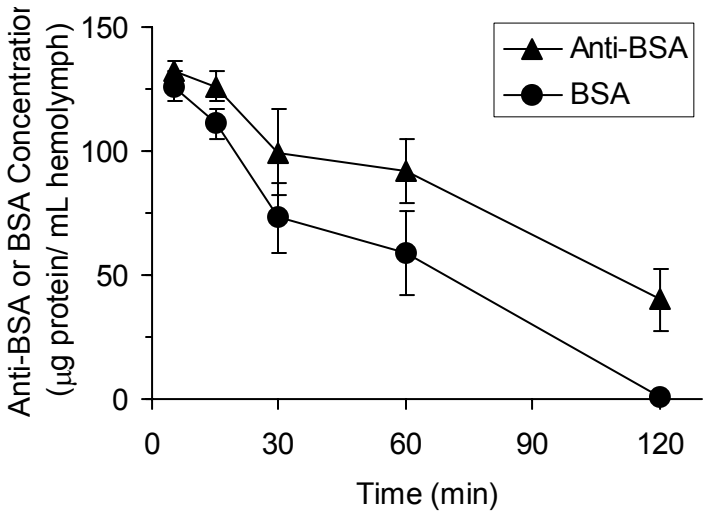


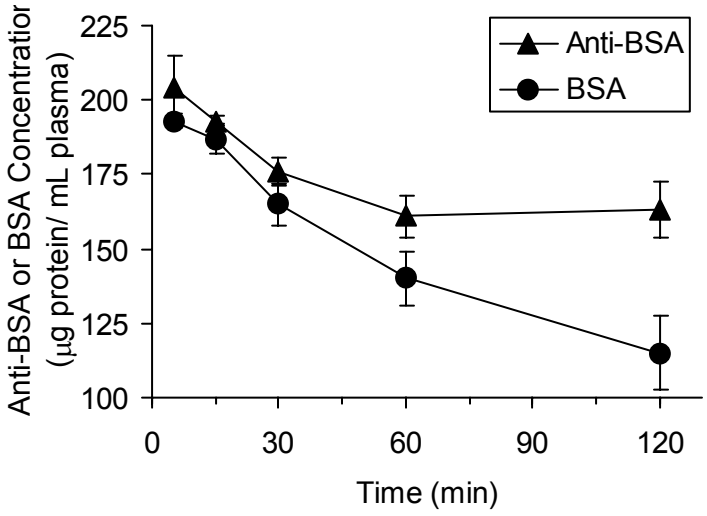
Heavy Chain (50 kDa)

Light Chain (25 kDa)

1 2 3 4 5


Anti-BSA or BSA Concentration (ng protein/ mL hemolymph)
A.
4000
3000
2000
1000
0
ND
ND
ND
0 h
Control
Anti-BSA
Control
Anti-BSA
8 h
16 h
B.
2000
1500
1000
500
0
ND
ND
ND
0 h
Control
BSA
Control
BSA
8 h
16 h
Treatment


Anti-BSA or BSA Concentration
(μg protein/ mL hemolymph)
150
100
50
0
0
30
60
90
120
Time (min)
Anti-BSA
BSA


Anti-BSA or BSA Concentration
(μg protein/ mL plasma)
225
200
175
150
125
100
0
30
60
90
120
Time (min)
Anti-BSA
BSA