Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Insect Physiology 51 (2005) 953–957
Journal of Insect Physiology
www.elsevier.com/locate/jinsphys

# Effects of soybean trypsin inhibitor on hypopharyngeal gland protein content, total midgut protease activity and survival of the honey bee (*Apis mellifera* L.)

Ramesh R. Sagili, Tanya Pankiw*, Keyan Zhu-Salzman

*Department of Entomology, Texas A&M University, College Station, TX 77843-2475, USA*

Received 30 December 2004; received in revised form 3 April 2005; accepted 6 April 2005

**Abstract**

Insecticidal properties of protease inhibitors have been established in transgenic plants. In the wake of continuous research and rapid development of protease inhibitors it is important to assess possible effects on beneficial insects like the honey bee (*Apis mellifera* L.). In this study, newly emerged caged bees were fed pollen diets containing three different concentrations (0.1%, 0.5% and 1% w:w) of soybean trypsin inhibitor (SBTI). Hypopharyngeal gland protein content, total midgut proteolytic enzyme activity of these bees, and survival were measured. Bees fed 1% SBTI had significantly reduced hypopharyngeal gland protein content and midgut proteolytic enzyme activity. There were no significant differences between control, 0.1% and 0.5% SBTI treatments. Bees fed a diet containing 1% SBTI had the lowest survival, followed by 0.5% and 0.1%, over a 30-day period. We concluded that nurse bees fed a pollen diet containing at least 1% SBTI would be poor producers of larval food, potentially threatening colony growth and maintenance.


*Keywords:* Soybean trypsin inhibitor; Hypopharyngeal gland; Midgut enzyme activity; Honey bee; *Apis mellifera*

## 1. Introduction

Plant protease inhibitor genes encode proteins that can inhibit insect protein digestive enzymes, resulting in starvation and even death of the insect (Michaud, 2000). Insect pests, however, are capable of evolving biotypes with adaptations to protease inhibitors that overcome or bypass toxic effects of protease inhibitors (Roush and Mackenzie, 1987). Beneficial insects, that act as pollinators, are additional co-evolutionary members among many plant–insect interactors (Delaplane and Mayer, 2000). The advent of genetic engineering techniques allows the transfer of plant insecticidal genes from one species to another (Gatehouse and Gatehouse, 1998).

Pollen is the most likely channel through which the honey bee will be exposed to transgenic protease inhibitors (Malone and Pham-Delegue, 2001). The honey bee has serine proteinases as digestive enzymes (Moritz and Crailsheim, 1987). Two serine trypsin endopeptidase inhibitors, bovine pancreatic trypsin inhibitor (BPTI) and soybean trypsin inhibitor (SBTI), known to be effective against a range of insect pests, are also toxic to adult honey bees at 1% wt:vol in sugar solution (Malone et al., 1995). There are very few published measurements of transgene expression levels in pollen; hence this limits our ability to design toxicity tests that mimic expression levels expected in the field. Plants can be protected from pests when protease inhibitors are expressed at approximately 1% of total soluble leaf protein (Hilder et al., 1987; Mcmanus et al., 1994). Protease inhibitor concentrations used in this study were estimates of the range of transgene product

*Corresponding author. Tel.: +1 979 458 0837; fax: +1 979 845 6305.
*E-mail address:* t-pankiw@tamu.edu (T. Pankiw).

0022-1910/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jinsphys.2005.04.003

concentrations a bee is expected to encounter while foraging. The lower concentration of 0.1% SBTI in pollen used in this study may represent a value closer to field relevance and the higher concentrations are unlikely to be encountered in the field and thus represent a worst case scenario.

We hypothesized that SBTI would have deleterious effects on honey bee protein digestion. In this study, we evaluated effects of SBTI on hypopharyngeal gland protein content, total midgut proteolytic enzyme activity and survival of adult honey bees. This study is the first to measure the effects of a protease inhibitor on hypopharyngeal gland protein content of honey bees. Hypopharyngeal glands are the brood food or protein-producing glands located in the head of worker honey bees called nurses (Patel et al., 1960). The diameters of the acini of hypopharyngeal glands in hive bees are largest when the hive bees are 8 days old (Crailsheim and Stolberg, 1989). Protein synthesis rates in hypopharyngeal glands are highest when the bees are 8–16 days old (Knecht and Kaatz, 1990). Pollen is the only source of protein for adult honey bees and consumption is necessary for gland development and protein production (Mohammedi et al., 1996). Insufficient pollen consumption early in life results in poor gland development and a shorter worker length of life (Maurizio, 1950; Haydak, 1970).

Protein digestion disruption affects hypopharyngeal gland protein production and consequently is expected to affect the ability of nurse bees to provision larvae with food. The combined effects of low larval food production and decrease in adult length of life could have serious consequences on colony population maintenance and growth. Hypopharyngeal glands in newly emerged bees treated with SBTI (0.1% and 1% w:v in sucrose solution) for 10 days have significantly reduced mean weights and acini diameter (Babendreier et al., 2005). Malone et al. (2004) reported no significant effects on survival and hypopharyngeal gland development of honey bees during evaluation of potential effects of a Bt toxin, a biotin binding protein and a protease inhibitor.

## 2. Materials and methods

Combs containing pupae were placed in an incubator maintained at 33 °C and 50% RH. Six hours later, newly emerged adults were placed in plexiglass-wire mesh cages (15 cm × 11 cm × 8 cm) and provisioned with gravity feeders containing sugar solution (40% w:v). Powdered pollen and SBTI dissolved in a small volume of sugar solution were mixed thoroughly. This uniform pollen mixture was packed into inverted vial caps and provided to the caged bees.

Cages were provisioned daily with fresh sucrose solution and pollen diet.

### 2.1. Hypopharyngeal gland protein quantification

The caged bees were fed three different concentrations (0.1%, 0.5% and 1% w:w) of SBTI (Sigma Aldrich product T-9003, St. Louis, MO, USA). Controls were handled in the same way but without the inhibitor. A randomized complete block design was used for this experiment. Eighty bees were randomly assigned to each cage and the cages were randomly assigned to treatments. The experiment was replicated four times for a total of 16 cages (4 treatments × 4 replications). On day 7, 10 bees were removed from each cage. Bees were cold anaesthetized, their hypopharyngeal glands removed and stored in Tris buffer at −80 °C prior to analysis. Frozen HP glands were homogenized and centrifuged at 10,000 rpm for 5 min. The supernatant was used to determine the protein concentration after Bradford (1976), described below.

#### 2.1.1. Bradford assay

Both hypopharyngeal glands from each bee were stored in 20 μl Tris Buffer pH 7.9 in 1.5 ml microcentrifuge tubes. Glands were homogenized using a homogenizer that tightly fitted onto each tube. Subsequently, tubes were centrifuged at 1000 rpm for 2 min. Supernatant from each tube was used for analysis. We used the 500-0202 Quick Start Bradford Protein Assay Kit 2, containing all reagents and dyes (Bio-Rad Laboratories, Hercules, CA, USA). Dye reagent was prepared by diluting 1 part Dye Reagent concentrate (Coomassie Brilliant Blue G-250) with 4 parts distilled water. We added 2 or 5 μl quantities of each sample to be analyzed to microcentrifuge tubes with 1 ml Bradford reagent. Tubes were vortexed to homogenize the contents, then incubated for 5 min at room temperature. Standard curves were prepared using bovine serum albumin (BSA). Protein absorbance was measured at 595 nm against blank reagent using a Beckman Spectrophotometer (Model #D4-640, Beckman Instruments, Inc., Columbia, MD, USA). Weight of protein (BSA) was plotted against the corresponding absorbance value to generate a linear regression equation (SAS PROC REG; SAS, 2000). To calculate micrograms of protein extracted from hypopharyngeal glands from measured absorbance values, we applied the linear regression equation generated from the BSA standard curve above.

Protein quantity was further analyzed using analysis of variance (ANOVA) (Sokal and Rohlf, 1995; SPSS, 2000). The data were log transformed prior to analysis to normalize the distribution (Sokal and Rohlf, 1995). Least significant difference (LSD) was used to signify between treatment differences. Beta or Type II error is more important in case of risk assessment studies. Hence

LSD which is a less conservative test for finding differences among treatments was used for multiple comparisons of treatments.

### 2.2. Total midgut proteolytic enzyme activity

The midguts of the same 7-day-old bees were excised from which the hypopharyngeal glands were removed above. Midguts were placed in centrifuge tubes containing 100 µl Tris-HCl buffer (pH 7.9) each and stored at −80 °C prior to further processing. Frozen guts were crushed, homogenized in Tris-HCl buffer (pH 7.9) and, centrifuged at 10,000 rpm for 5 min to remove particulate matter. The supernatant was analyzed for total midgut proteolytic enzyme activity (casenolytic activity) as described by Michaud et al. (1995).

Five microlitres of supernatant was used for each reaction. Twenty microlitres of assay buffer (0.1 M Tris-HCl, pH 7.9) and 60 µl of 2% (w/v) azocasein diluted in assay buffer were added, respectively, to the supernatant and incubated for 6 h at 37 °C. To remove the residual azocasein after proteolysis, 300 µl of 10% (w:v) TCA was added to each mixture and centrifuged for 5 min at 10,000 rpm. Three hundred and fifty microlitres of supernatant was added to 200 µl of 50% ethanol in water, and the absorbance of this mixture was measured at 440 nm using a Beckman DU 64 spectrophotometer. Absorbance of the sample without proteolysis (no incubation) was used to zero the machine. Total midgut proteolytic activity was expressed in terms of $OD_{440}$. Data were log transformed prior to analysis to meet assumptions of ANOVA (Sokal and Rohlf, 1995). Mean total midgut proteolytic enzyme activities from each treatment were analyzed using ANOVA and LSD (Sokal and Rohlf, 1995; SPSS, 2000). Correlation analysis (parametric) using SPSS was performed to measure the strength of linear association between midgut enzyme activity and hypopharyngeal gland protein quantity.

### 2.3. Survival analysis

Daily, for 30 days, the number of dead bees per cage was recorded and bodies removed. Survival curves were generated by plotting the number of surviving bees against days from initiation of the experiment. Kaplan–Meier survival curves were used to plot and interpret the survival data (Le, 1997). Survival curves were compared using log rank tests (Allison, 1998; SAS, 2000). A Cox proportional hazard (PH) model was used to model the survival data using SPSS. Bees that survived up to the termination of the experiment (day 30) and those that were removed from the cages for the Bradford Assay and midgut enzyme activity analysis were treated as censored cases.

## 3. Results

### 3.1. Hypopharyngeal gland development

Dose-dependent effects of SBTI on amount of extractable protein from adult hypopharyngeal glands are summarized in Fig. 1. Replicates were not significantly different and therefore pooled for subsequent analysis ($F_{3,156} = 0.976$, $P = 0.42$). Significant differences were observed between 1% SBTI and remaining diets, i.e. 0.1%, 0.5% and control (ANOVA, $F_{3,156} = 6.4$, $P < 0.003$). SBTI diet of 1% had significantly lower hypopharyngeal gland protein quantity than all other diets ($P < 0.003$).

### 3.2. Total midgut protease activity

Overall, 1% SBTI resulted in significantly lower midgut protease activity compared to all the other doses. Effects of SBTI on the total midgut protease activity of adult bees fed different doses of SBTI in a pollen diet are summarized in Fig. 2. The four replicates were pooled because there were no significant differences between them ($F_{3,156} = 0.654$, $P = 0.582$). Pairwise comparisons of the SBTI treatments showed that there were significant differences among treatments (ANOVA, $F_{3,156} = 237.5$, $P < 0.0001$). SBTI treatment of 1% had significantly lower midgut protease activity ($P < 0.0001$). There were no significant differences between control, 0.1% and 0.5% SBTI. Midgut enzyme activity explained 31.1% of the variation in hypopharyngeal gland protein quantity (correlation analysis $\rho = 0.311$, $P = 0.01$).

### 3.3. Survival

Kaplan–Meier survival curves (SAS) were used to plot survival data. Log-rank tests indicated that there were

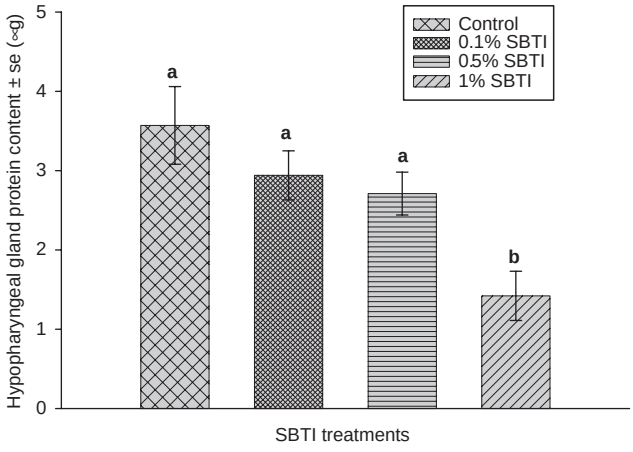


Fig. 1. Mean hypopharyngeal gland protein quantities of bees (±SE) fed with different concentrations of soybean trypsin inhibitor in pollen. Different letters indicate significant differences among the treatments ($P < 0.0001$).

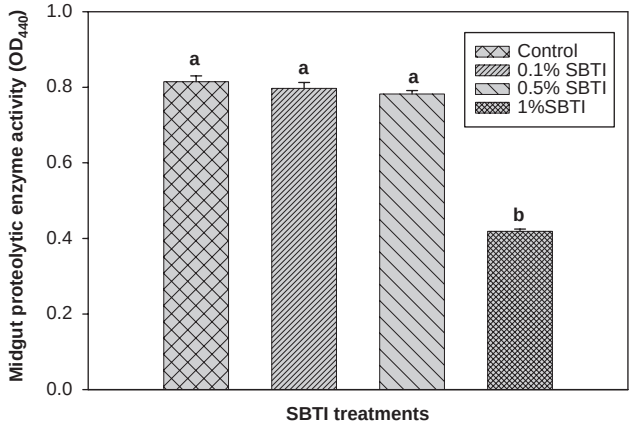


Fig. 2. Mean midgut proteolytic enzyme activities of bees (+SE) fed with different concentrations of soybean trypsin inhibitor in pollen. Different letters indicate significant differences among the treatments ($P<0.0001$).

significant differences in survival among bees that were fed different doses of SBTI ($\chi^2 = 87.27$, df = 3 and $P<0.0001$). Kaplan–Meier curves showed that bees fed with 1% SBTI concentration had lowest survival, followed by 0.5% and 0.1% SBTI. The control had the highest survival. Cox proportional hazard (PH) model was used to model the survival data using SPSS. Cox regression is a method for modeling time-to-event data in the presence of censored cases. SBTI treatment had a significant effect on survival ($\chi^2 = 81.75$, df = 3 and $P<0.0001$). Hazard ratio ($e^{\beta}$) was 1.667. The hazard ratio statistic $e^{\beta}$, was transformed to a more meaningful statistic indicating that with each dosage increase of SBTI used in this study, mortality increased by 66.7% over the 30-day experiment period. This meaningful statistic was obtained by subtracting 1.0 from the risk ratio and multiplying by 100.

## 4. Discussion

In this study, hypopharyngeal gland protein quantity, midgut protease activity and survival were significantly lower when bees were fed 1% SBTI in pollen, strongly suggesting a dose-dependent effect. Nurse bees ingesting SBTI at higher concentrations may be poor producers of brood food as a result of poor development of hypopharyngeal glands. Babendreier et al. (2005) reported that bees fed with 1% SBTI (w:v in sucrose solution) treatment did not rear any brood while there was brood rearing in other treatments.

Hypopharyngeal glands in bees fed with 0.1% or 1% SBTI (w:v in sucrose solution) are lighter in weight and have smaller acini when compared to controls (Babendreier et al., 2005). These results cannot be directly compared to our results as the bees in the above study were fed SBTI in sucrose solution instead of pollen as in our study. Malone (2004) reported that there was no effect of three transgene products a Bt toxin, a biotin-binding protein (avidin) and a protease inhibitor (aprotinin) on the hypopharyngeal gland development of bees.

Bees fed 1% SBTI had significantly lower levels of midgut protease activity compared to controls. Similar results were reported by Burgess et al. (1996), where bees fed with a highest dose of 1% SBTI had significantly lower levels of three endopeptidases, chymotrypsin, elastase and trypsin. Effects on survival may be attributed to a certain extent to lowered midgut protease activity levels. Apart from reduced midgut proteolytic enzyme activity there may be some other factors which are responsible for the reduced hypopharyngeal gland protein biosynthesis and bee survival, because midgut proteolytic enzyme activity accounted for only 31% of hypopharyngeal gland extractable protein. Burgess et al. (1996) reported that in addition to lowered endopeptidase levels, decreased bee longevity may be also explained by additional metabolic cost incurred because of compensatory hyperproduction of proteolytic enzymes, to compensate for deactivation of enzymes by the protease inhibitor. Hence we may infer that additional metabolic cost incurred as a result of compensatory hyperproduction is the other major factor responsible for decrease in hypopharyngeal gland protein quantity apart from reduced midgut enzyme activity.

The higher concentration of SBTI used in this study or in other similar studies is unlikely to be encountered by the bees in the field and hence represent a worst case scenario. The lower concentration of SBTI (0.1%) used represents a value closer to field relevance if it is expressed in the pollen. Expression levels of protease inhibitors like SBTI also depend upon the type of promoter used. In our experimental design the bees did not rear any brood which is considered to be a factor stimulating hypopharyngeal gland development. But this criterion does not affect our study as we are comparing the hypopharyngeal gland development between treatments receiving different concentrations of SBTI, keeping all other factors constant. Also, Malone et al. (2004) observed measurable hypopharyngeal gland development in caged bees that were used in a study to evaluate potential impacts of transgene products on hypopharyngeal gland development.

In conclusion, this study has revealed that SBTI at 1% of pollen diet can negatively impact the hypopharyngeal gland development, midgut protease activity and survival of honey bees. In contrast, it also showed that lower doses of SBTI were not deleterious to adult bees. Because honey bee larvae are completely dependent on the hypopharyngeal gland secretions of nurse bees for their nutritional needs, the deleterious effects of SBTI on hypopharyngeal glands could negatively impact colony growth and maintenance. However, the threshold

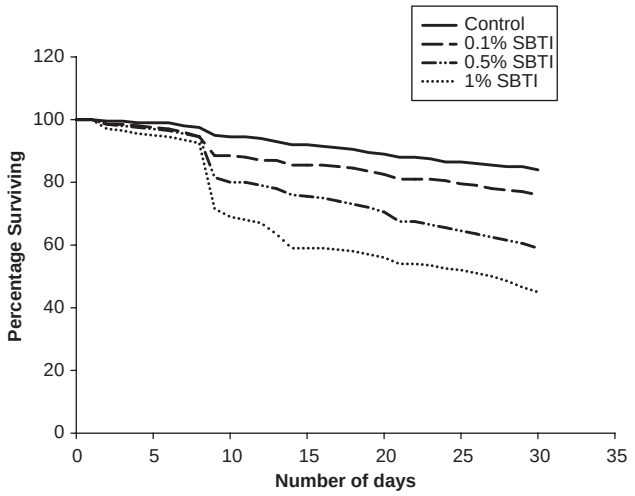


Fig. 3. Survival of bees fed with different concentrations of soybean trypsin inhibitor in pollen.

response shown in this study strongly suggests that pollen diets containing less than 1% SBTI are tolerated and unlikely to adversely affect colonies (Fig. 3).

## Acknowledgements

This research was supported by funds from USDA/NRI 2004-35302-15031, USDA 58-6204-093 and 58-6204-1-009 to TPankiw, and a scholarship to RRSagili from The Foundation for the Preservation of Honey Bees. Treatment of bees complied with USA laws and regulations.

## References

Allison, P.D., 1998. Survival Analysis Using the SAS System. A Practical Guide. SAS Institute Inc., Cary.

Babendreier, D., Kalberer, N.M., Romeis, J., Fluri, P., Mulligan, E., Bigler, F., 2005. Influence of Bt-transgenic pollen, Bt-toxin and protease inhibitor (SBTI) ingestion on development of the hypopharyngeal glands in honeybees. Apidologie, in press.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Burgess, E.P.J., Malone, L.A., Christeller, J.T., 1996. Effects of two proteinase inhibitors on the digestive enzymes and survival of honey bees (*Apis mellifera*). Journal of Insect Physiology 42, 823–828.

Crailsheim, K., Stolberg, E., 1989. Influence of diet, age and colony condition upon intestinal proteolytic activity and size of the hypopharyngeal glands in the honeybee (*Apis mellifera* L.). Journal of Insect Physiology 35, 595–602.

Delaplane, K.S., Mayer, D.F., 2000. Crop Pollination by Bees. CABI Publishing, Cambridge.

Gatehouse, A.M.R., Gatehouse, J.A., 1998. Identifying proteins with insecticidal activity: use of encoding genes to produce insect-resistant transgenic crops. Pesticide Science 52, 165–175.

Haydak, M.H., 1970. Honey bee nutrition. Annual Review of Entomology 15, 143–156.

Hilder, V.A., Gatehouse, A.M.R., Sheerman, S.E., Barker, R.F., Boulter, D., 1987. A novel mechanism of insect resistance engineered into tobacco. Nature 330, 160–163.

Knecht, D., Kaatz, H.H., 1990. Patterns of larval food production by hypopharyngeal glands in adult worker honey bees. Apidologie 21, 457–468.

Le, C.T., 1997. Applied Survival Analysis. Wiley-Interscience Publication, New York.

Malone, L.A., Pham-Delegue Minh-Ha, 2001. Effects of transgene products on honey bees (*Apis mellifera*) and bumblebees (*Bombus* sp.). Apidologie 32, 287–304.

Malone, L.A., Giacon, H.A., Burgess, E.P.J., Maxwell, J.Z., Christeller, J.T., Laing, W.A., 1995. Toxicity of trypsin endopeptidase inhibitors to honey bees (Hymenoptera: Apidae). Journal of Economic Entomology 88, 46–50.

Malone, L.A., Todd, J.H., Burgess, E.P.J., Christeller, J.T., 2004. Development of hypopharyngeal glands in adult honey bees fed with a Bt toxin, a biotin binding protein and a protease inhibitor. Apidologie 35, 655–664.

Maurizio, A., 1950. The influence of pollen feeding and brood rearing on the length of life and physiological condition of the honeybee. Bee World 31, 9–12.

Mcmanus, M.T., White, D.W.R., Mcgregor, P.G., 1994. Accumulation of a chymotrypsin inhibitor in transgenic tobacco can affect the growth of insect pests. Transgenic Research 3, 50–58.

Michaud, D., 2000. Recombinant Protease Inhibitors in Plants. Eurekah, Georgetown.

Michaud, D., Cantin, L., Vrain, T.C., 1995. Carboxy-terminal truncation of oryzacystatin-II by oryzacystatin-insensitive insect digestive proteinases. Archives of Biochemistry and Biophysics 322, 469–474.

Mohammedi, A., Crauser, D., Paris, A., Le Conte, Y., 1996. Effect of a brood pheromone on honeybee hypopharyngeal glands. Comptes Rendus de l' Académie des Sciences Paris, Sciences de la vie 319, 769–772.

Moritz, B., Crailsheim, K., 1987. Physiology of protein digestion in the midgut of the honeybee (*Apis mellifera* L.). Journal of Insect Physiology 33, 923–931.

Patel, N.G., Haydak, M.H., Gochnauer, T.A., 1960. Electrophoretic components of the proteins in honeybee larval food. Nature 186, 633–634.

Roush, R.T., Mackenzie, J.A., 1987. Ecological genetics of insecticide and acaricide resistance. Annual Review of Entomology 32, 361–381.

SAS, 2000. The SAS System Version 8.01. SAS Institute Inc., Cary.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. The Principles and Practice of Statistics in Biological Research, third ed. W.H. Freeman and Company, New York.

SPSS, 2000. SPSS for Windows, Version 11.5. SPSS Inc., Chicago.