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

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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

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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