



Effects of Two Proteinase Inhibitors on the Digestive Enzymes and Survival of Honey Bees (*Apis mellifera*)

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Two endopeptidase inhibitors, BPTI (bovine pancreatic trypsin inhibitor) and SBTI (Kunitz soybean trypsin inhibitor), were found to significantly reduce the longevity of adult honey bees (*Apis mellifera* L.) fed the inhibitors *ad lib* in sugar syrup at 1.0%, 0.5% or 0.1%, but not at 0.01% or 0.001% (w:v). Bees were taken from frames at emergence, kept in cages at 33°C, and provided with a pollen/protein diet, water and syrup. *In vivo* activity levels of three midgut endopeptidases (trypsin, chymotrypsin and elastase) and the exopeptidase leucine aminopeptidase (LAP) were determined in bees fed either BPTI or SBTI at 1.0%, 0.3% or 0.1% (w:v) at two time points: the 8th day after emergence and when 75% of bees had died. LAP activity levels increased significantly in bees fed with either inhibitor at all concentrations. At day 8, bees fed BPTI at all concentrations had significantly reduced levels of trypsin, chymotrypsin and elastase. At the time of 75% mortality, bees fed BPTI at each concentration had reduced trypsin levels, but only those fed the inhibitor at the highest dose level had reduced chymotrypsin or elastase activity. At both time points, only bees fed SBTI at the highest concentration had lowered trypsin, chymotrypsin and elastase activities. Copyright © 1996 Elsevier Science Ltd

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INTRODUCTION

Proteinase inhibitors from a variety of plant and animal sources have been shown to reduce the growth and survival of a range of insects when added to their food (Steffens *et al.*, 1978; Gatehouse *et al.*, 1979; Burgess *et al.*, 1991, 1994; Johnston *et al.*, 1991, 1993, 1995). They act by inhibiting digestion and effectively starving the insects, although their exact modes of action are as yet uncertain (Burgess and Gatehouse, in press). They bind directly with and deactivate proteinases in the gut (Laskowski and Kato, 1980; Ryan, 1989), thereby reducing the insect's digestive capacity. The insect may also respond to this binding, via a secretagogue mechanism, with a hyperproduction of proteinases (Broadway and Duffey, 1986; Larocque and Houseman, 1990; Burgess *et al.*, 1991; Dymock *et al.*, 1992). If the proteinase

inhibitors are sufficiently potent, or being ingested at sufficiently high levels, these additional digestive enzymes may also be bound and deactivated. Additionally, hyperproduction itself may stress the insect's metabolism sufficiently to contribute to the detrimental effects observed in insects fed with proteinase inhibitors. Some insects may also respond to the ingestion of proteinase inhibitors with the induction of a new proteinase activity that is insensitive to the inhibitor (Jongsma *et al.*, 1995).

Because of their effectiveness against pest insects, and the fact that they are the products of single genes, a number of proteinase inhibitors have been incorporated into transgenic plants, where they have successfully conferred pest resistance (Hilder *et al.*, 1987; Johnson *et al.*, 1989; Boulter *et al.*, 1990). Initiatives to identify inhibitors effective against a range of pests and suitable for incorporation into a wide array of crop plants are under way in many parts of the world (Ryan, 1990; Gatehouse *et al.*, 1992).

As with any new method of insect control, the likely impact of transgenic plants containing proteinase inhibitor genes on non-target and beneficial insects, particularly pollinators such as honey bees, needs to be

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assessed. Bees may be affected by transgenic plants in two ways. Firstly, direct ingestion of the gene product in pollen or nectar may have an effect on the bee although, as nectar contains only small quantities of amino acids (Baker and Baker, 1977), it is far less likely to carry the gene product than pollen which has a large protein component (Stanley and Linskens, 1974). Because of the social nature of honey bees, transgenic pollen collected by a foraging bee may be stored in the hive and subsequently ingested by many other adult and older larval bees and also transferred among adults via trophallaxis (Herbert, 1992). Secondly, expression of a foreign gene may result in pleiotropic effects in the transgenic plants that may make them less attractive or nutritious to bees. For example, Picard-Nizou *et al.* (1995) found that some transgenic rape plants carrying a chitinase gene produced a greater volume of nectar with a higher sucrose content than unmodified plants. As the behaviour of pollinating insects such as bees relies on a series of complex sensory responses (Gary, 1992), even minor alterations in plant biochemistry may alter bee behaviour.

Two inhibitors of trypsin endopeptidases, bovine pancreatic trypsin inhibitor (BPTI) and Kunitz soybean trypsin inhibitor (SBTI), have high *in vitro* binding affinities for the major honey bee digestive endopeptidases and high doses (1% w:v) of these inhibitors are toxic when fed in sugar syrup to adult honey bees (Malone *et al.*, 1995). However, lower doses (0.1–0.0001% w:v) of Bowman-Birk soybean trypsin inhibitor (SBBI) do not cause significant bee mortality, even though this inhibitor has a high binding affinity for bee trypsin *in vitro* and reduces the activity of this enzyme *in vivo* (Belzunces *et al.*, 1994). In this paper we report on a study designed to determine which doses of BPTI and SBTI may be fed to honey bees without a reduction in survival, and to explore the relationships between the effects of these inhibitors on bee gut proteolytic activity and on bee survival. Caged adult bees were fed BPTI and SBTI at five different dose levels (1.0–0.001% w:v) and their survival measured. In a second experiment, bees were fed the two inhibitors at three different doses (1.0–0.1% w:v) and the *in vivo* levels of four digestive peptidases were measured at 8 days after emergence and at the time at which 75% of bees had died.

MATERIALS AND METHODS

Honey bees of the yellow Italian race, *Apis mellifera ligustica* (Hymenoptera: Apidae), were obtained from a local commercial bee breeder and kept in our apiary at the Mt Albert Research Centre, Auckland, New Zealand. Frames containing capped brood were incubated in darkness at 33°C for up to 2h, and newly-emerged adult bees were collected and assigned randomly to 48 groups of 20 for the survival experiment or 24 groups of 25 for enzyme analysis. Each group was placed in a cage of 9 × 8 × 6 cm (internal dimensions), based on a design reported by Kulinčević *et al.* (1973) and constructed from

plywood (4 sides, with holes for gravity feeders) and stainless steel mesh (2 sides). Each cage was kept in an incubator at 33°C and bees were provided, *ad libitum*, via gravity feeders, with water and a 60% (w:v) sucrose solution. In addition, a dietary supplement consisting of pollen (0.33 parts), sodium caseinate (0.08 parts), brewer's yeast (0.16 parts) and sucrose (0.43 parts) mixed with water to a paste, was placed in each cage. The pollen used was bee-collected from white clover and kept frozen until required. Water, sucrose solution and pollen supplement were replaced at 2-day intervals. SBTI (from Sigma Chemical Co., St. Louis, MO, U.S.A.) and BPTI (from Trace Biosciences NZ Ltd, Hamilton, NZ) were fed to bees in separate tests, both dissolved in the sucrose solution.

Two separate experiments were carried out: one to determine the effects of each of the two proteinase inhibitors on bee survival and one to measure their effects on bee gut proteolytic enzymes.

For the first experiment, two tests, one for BPTI and one for SBTI, consisting of six treatments each were run: 1.0%, 0.5%, 0.1%, 0.01%, 0.001% (w:v) BPTI or SBTI in sucrose solution, and sucrose solution with no addition as a control. Four consecutive blocks, each consisting of six cages receiving one of each of the above treatments were run (i.e. 24 cages in total per test). A randomised complete block design was used. Twenty bees were randomly assigned to each cage, and cages randomly assigned to treatments (i.e. 80 bees per treatment). Bees used in a single block were all derived from a single frame. All the bees used in this experiment were from a single colony. All bees were checked daily for survival and the longevity of each bee recorded in days from commencement of each test. For each test, data from the four blocks were combined as any differences between the blocks of any given treatment appeared to be due to random variation. Mean longevities of bees from each of the six treatments were compared by analysis of variance using Tukey's LSD. As both tests were run at the same time, the control data from each were combined for comparison with the proteinase inhibitor treatment data (see Fig. 1).

The second experiment also consisted of two tests (BPTI and SBTI), each with four different treatments: 1.0%, 0.3%, 0.1% BPTI or SBTI in sucrose solution, and sucrose solution with no addition. For this experiment, 25 bees were assigned to each cage and three consecutive blocks, each consisting of the four different treatments (i.e. 75 bees per treatment and a total of 12 cages per test) were run in a randomised complete block design as described above. The bees used in this experiment were all from a different single colony from that used in the first. All bees were checked daily for survival. Five live bees were removed for enzyme analysis from each cage eight days after commencing each block and a further five removed at the time when these were the only survivors remaining in the cage (i.e. at 75% mortality). Digestive enzyme activities were determined by cold-

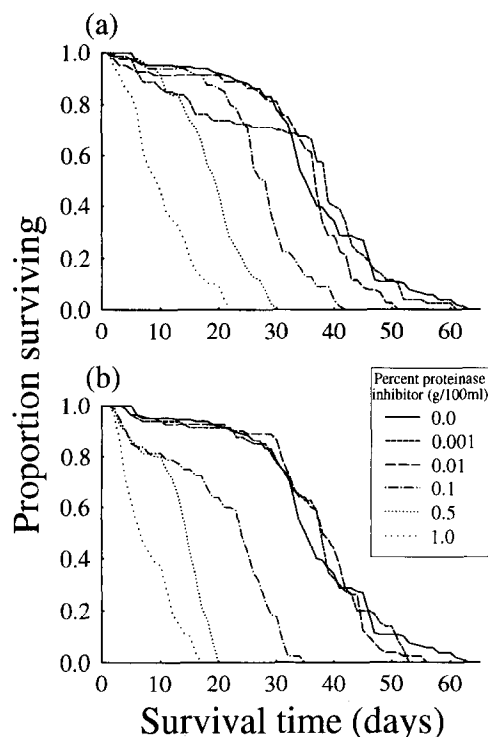


FIGURE 1. Effects of different doses of proteinase inhibitors on survival of adult honey bees. (a) Survival of bees fed bovine pancreatic trypsin inhibitor (BPTI) at five different concentrations; (b) survival of bees fed Kunitz soybean trypsin inhibitor (SBTI) at five different concentrations. Comparison of mean longevity of bees, by analysis of variance using Tukey's LSD, showed that consumption of either inhibitor at 1, 0.5 or 0.1% (w:v) significantly reduced bee longevity ($P < 0.001$).

anaesthetising the bees, dissecting out their midguts and storing them at -80°C for subsequent assay. Activities of three endopeptidases (chymotrypsin, elastase [*N*-succinyl-L-alanyl-L-pro-L-leu-*p*-nitroanilide-hydrolysing] and trypsin) and one exopeptidase (leucine amino peptidase [LAP]) were measured as described by Christeller and Shaw (1989) and Christeller *et al.* (1992). For each test, data from the three blocks were combined as there were no significant differences among them. Because of the skewed nature of the data, logs of values were analysed. Mean enzyme activities for bees from each treatment were compared by analysis of variance using Tukey's LSD.

RESULTS

The effects of different doses of BPTI or SBTI on the survival of caged honey bees are shown in Fig. 1. Analysis of variance (using Tukey's LSD) indicates that bees fed either BPTI or SBTI as 1.0%, 0.5% or 0.1% (w:v) of their sugar syrup have significantly reduced longevity compared with the controls ($P < 0.001$). At these three dose levels, there is a dose-dependent reduction in bee longevity with both proteinase inhibitors.

Figures 2 and 3 show the effects of three different doses of BPTI or SBTI on the *in vitro* activity levels of four different bee digestive proteinases at two different

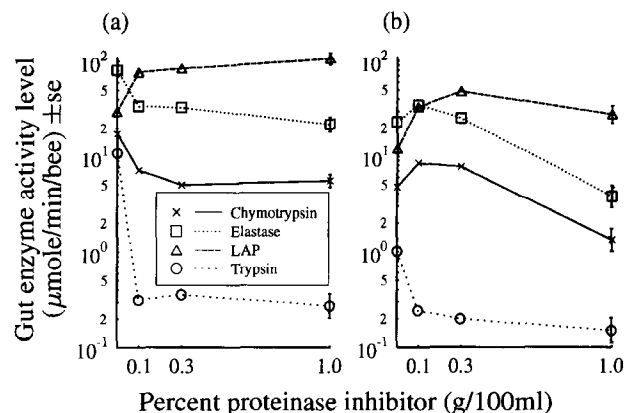


FIGURE 2. Effects of different doses of bovine pancreatic trypsin inhibitor (BPTI) on *in vivo* activities of four honey bee digestive proteinases (chymotrypsin, elastase, leucine aminopeptidase [LAP] and trypsin). (a) Enzyme activities in adult bees examined after 8 days of feeding on bovine pancreatic trypsin inhibitor (BPTI); (b) enzyme activities in BPTI-fed bees examined when 75% of bees had died. Enzyme activity levels in control bees fed with plain sugar syrup are plotted on the y-axis. Activities are shown on a logarithmic scale. After using Bartlett's test for homogeneity of variance (Miller, 1986) on this scale, we based S.E.s on an estimate of standard deviation that was pooled over all levels of inhibitor.

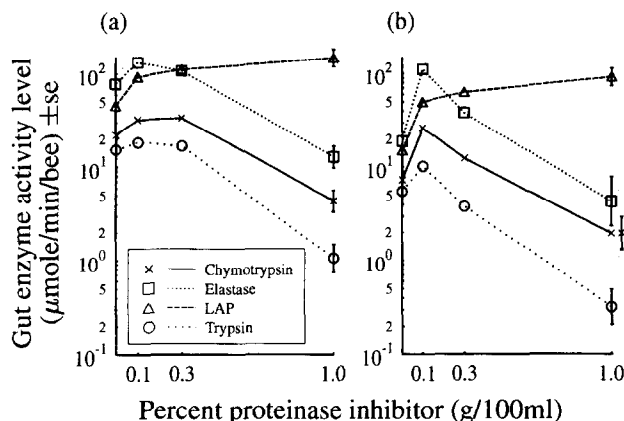


FIGURE 3. Effects of different doses of Kunitz soybean trypsin inhibitor (SBTI) on *in vivo* activities of four honey bee digestive proteinases (chymotrypsin, elastase, leucine aminopeptidase [LAP] and trypsin). (a) Enzyme activities in adult bees examined after 8 days of feeding on SBTI; (b) enzyme activities in SBTI-fed bees examined when 75% of bees had died. The S.E. for chymotrypsin is offset for clarity. Enzyme activity levels in control bees fed with plain sugar syrup are plotted on the y-axis. Activities are shown on a logarithmic scale. After using Bartlett's test for homogeneity of variance (Miller, 1986) on this scale, we based S.E.s on an estimate of standard deviation that was pooled over all levels of inhibitor.

time points. In both experiments, the control activity levels of each proteinase were higher at day 8 than at the time when 75% of bees had died.

The patterns of enzyme activity level response in bees fed different doses of BPTI differed between day 8 and the time of 75% mortality [Fig. 2(a, b)]. At day 8, levels of the exopeptidase, LAP, were significantly higher in all three groups of dosed bees (0.1%, 0.3% and 1.0%) than in the controls ($P < 0.05$) [Fig. 2(a)]. However, the levels of the three endopeptidases, chymotrypsin, elastase and

trypsin, were significantly lower in all BPTI-dosed bees than in the controls ($P < 0.05$) [Fig. 2(a)]. For each enzyme, there were no significant differences in activity levels among the three different dose groups.

At the time of 75% mortality [Fig. 2(b)], levels of LAP were once again significantly higher in the BPTI-dosed bees than in the control bees ($P < 0.05$). Levels of trypsin were significantly lower in all BPTI-dosed bees than in the controls ($P < 0.05$). In contrast to the day 8 results, however, chymotrypsin and elastase levels were significantly lower than in the controls only in those bees receiving the highest dose (1.0%) of BPTI ($P < 0.05$) [Fig. 2(b)].

In contrast to the BPTI-fed bees, the patterns of enzyme level changes in the bees fed SBTI were similar for both the day 8 data and that recorded at the time of 75% mortality [Fig. 3(a and b)]. Only those bees fed the highest dose (1.0%) of SBTI had significantly lower levels of the three endopeptidases, chymotrypsin, elastase and trypsin ($P < 0.05$). However, all three doses of SBTI resulted in significantly elevated levels of the exopeptidase, LAP ($P < 0.05$).

DISCUSSION

Within the life cycle of the honey bee, protein consumption is most marked in newly-emerged adults (Hagedorn and Moeller, 1967) and correspondingly high levels of proteolytic activity have been recorded in the guts of such bees (Dahlmann *et al.*, 1978; Moritz and Crailsheim, 1987). This period of protein digestion is thought to be necessary for proper development of the hypopharyngeal glands in these young, 'nurse' bees. The secretions of these glands are an important component of the 'larval jelly' or 'brood food' with which the nurses feed the larvae. By the time an adult bee ceases to be a nurse and begins to forage (after about three weeks in summer), protein consumption and proteolytic activity in the gut have dropped considerably (Moritz and Crailsheim, 1987; Jimenez and Gilliam, 1989). Comparisons of bees kept in hives and in cages have shown a similar pattern of feeding and digestion (provided pollen is supplied), but with the peak of proteolytic activity occurring earlier, at about day 3 rather than day 8 (Crailsheim and Stolberg, 1989), and a steady decline in enzyme levels thereafter (Gilliam *et al.*, 1988). Our results are in agreement with these observations; levels of each of the four peptidases in the control bees were lower at the time of 75% mortality than at the 8th day after emergence (Figs 2 and 3).

In our study, bees fed BPTI at doses shown to cause a significant reduction in longevity also had significantly lowered levels of activity of the three endopeptidases, trypsin, chymotrypsin and elastase, at day 8, a time when proteolytic activity would still be important for normal bee development. Although we did not obtain direct evidence for the formation of enzyme-inhibitor complexes, the simplest explanation for these observations is that

BPTI is disrupting the activity of these enzymes, perhaps via direct inhibition, and that this disruption, especially during this early stage of adult development, is sufficient to reduce bee life expectancy. The effects of BPTI on chymotrypsin and elastase are less marked in older bees, with only the highest dose of inhibitor causing a reduction in enzyme activity [Fig. 2(b)]. This may reflect the relatively greater *in vitro* binding affinity of BPTI for bee trypsin than for the other two endopeptidases (Malone *et al.*, 1995). However, since protein digestion is less important at this later stage of the bee's life cycle than at the earlier time point, BPTI may have already had its greatest impact on the bee's life expectancy and the enzyme levels observed at this time may not be of such significance in determining bee longevity.

Levels of the exopeptidase, LAP, were significantly higher in bees fed either BPTI or SBTI at any dose level than in the controls (Figs 2 and 3). As both proteinase inhibitors are endopeptidase inhibitors which demonstrate no binding affinity for LAP, the lack of an inhibitory effect on this enzyme is not surprising and it is unlikely that LAP activity would increase in direct response to the presence of an endopeptidase inhibitor. Similar increases in LAP activity have been observed in black field crickets fed potato proteinase inhibitor 2 (Burgess *et al.*, 1991, 1994). The observation that LAP activity increased in bees fed SBTI at 0.1% and 0.3% [Fig. 3(a, b)] in which there was no significant alteration in endopeptidase activities shows that this effect does not necessarily occur in response to reduced digestive capacity. One possible explanation is that the insects are responding to ingestion of the endopeptidase inhibitors with a generalised hyperproduction of proteases, but that this increase in activity can only be measured for the exopeptidase which is not a direct target for the inhibitors. Measurement of enzyme-inhibitor complexes within the bee gut would provide a means of testing this hypothesis. Thus, the *in vivo* enzyme analysis presented here suggests that the mode of action of these two proteinase inhibitors is more complex than a simple, direct inhibition of gut proteolytic activity.

In contrast to BPTI, SBTI affected trypsin, chymotrypsin and elastase similarly at both time points, perhaps reflecting the relatively uniform *in vitro* binding affinities of these three enzymes for SBTI, whereas BPTI binds preferentially with trypsin. SBTI appears to be less effective than BPTI as an inhibitor of bee endopeptidases, as only the highest dose levels of this proteinase inhibitor altered the activities of these enzymes [Fig. 3(a, b)], whereas all doses of BPTI resulted in enzyme level changes [Fig. 2(a, b)]. Despite this, bee survival was significantly reduced when bees were fed SBTI at the lowest dose examined in the enzyme assays (0.1%) (Fig. 1). Thus, reductions in the longevity of bees fed SBTI cannot be attributed solely to lowered endopeptidase levels. The observed levels of endopeptidase activity in bees fed 0.3% or 0.1% SBTI, although not significantly different from the controls, may represent the net result of a

deactivation of enzyme by the inhibitor and a compensating hyperproduction, so that decreased bee longevity may be explained by the additional metabolic cost of these processes. Increased LAP production may have also contributed to this cost. The converse association between longevity and proteinase inhibition has been noted in caged bees fed SBBI (Belzunces *et al.*, 1994), where trypsin activity levels were lowered significantly but bee survival was not affected. These results are not directly comparable with the current study, however, as the SBBI experiments were conducted with older, foraging bees which were kept in cages without protein food, whereas we used newly-emerged bees fed with a pollen-containing diet.

In conclusion, our results indicate that both BPTI and SBTI are significantly toxic to adult honey bees only when ingested at concentrations of 0.1% or more (w:v) in sugar syrup and not when ingested at 0.01% (w:v) or less. When considering the implications of this result for the development of transgenic pest-resistant plants containing proteinase inhibitor genes, it is important to take into account the differences between the experimental set-up used and the field situation. Bees in the field will not be as well-fed or inactive as those kept in cages and so they may succumb more readily to proteinase inhibitors. On the other hand, it is extremely unlikely that they will receive a continuous supply of inhibitor in nectar, but are much more likely to ingest a smaller quantity in the pollen that is a major part of their diet only in the early stages of adult life. Proteinase inhibitors have been shown to protect plants from pest attack when expressed as approximately 1% of total soluble leaf protein (Hilder *et al.*, 1987; McManus *et al.*, 1994) or 50–100 µg/g of leaf tissue (Johnson *et al.*, 1989). However, inhibitor levels have not been measured in the pollen of such plants, so that a meaningful comparison with the results presented here is not possible at this stage. That low levels of the inhibitors studied here were not toxic to bees suggests that the development of transgenic pest-resistant plants, which contain proteinase inhibitor genes and are safe to honey bees, is feasible. Furthermore, the problem of bee exposure to gene products in pollen may be diminished by careful selection of tissue-specific promoters, e.g. CaMV 35S which apparently allows expression in all tissues except pollen (Twiss *et al.*, 1989).

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