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### DIETARY MIXTURES OF CYSTEINE AND SERINE PROTEINASE INHIBITORS EXHIBIT SYNERGISTIC TOXICITY TOWARD THE RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM*

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- Abstract**—1. Combinations of a cysteine proteinase inhibitor (CPI) and serine proteinase inhibitors (SPI) in wheat germ diets were toxic to larvae of the red flour beetle, *Tribolium castaneum*, when tested at levels where individual inhibitors were nontoxic.
2. Mixtures of 0.1% (w/w) CPI (E-64) plus 1% of either of three plant SPIs (soybean Kunitz trypsin inhibitor, soybean Bowman-Birk trypsin-chymotrypsin inhibitor, or lima bean trypsin inhibitor) inhibited *T. castaneum* growth, resulting in 82-97% reduction in larval weight gain 17 days after hatching and 40-60% mortality.
3. Supplementation of diet containing 0.1% E-64 plus 1% soybean Kunitz trypsin inhibitor (STI) with a mixture of amino acids at 0.7% caused a partial reversal of the growth inhibition, with 91% of the larvae surviving.
4. Diet containing 0.1% E-64 plus either 5 or 10% STI resulted in 100% mortality of the larvae during the first or second instar.
5. Addition of a mixture of amino acids at 20% to the 0.1% E-64 plus 10% STI diet allowed 89% of the larvae to develop into adults.
6. The synergism between different classes of proteinase inhibitors in the insect's diet that enhances growth inhibition and toxicity demonstrates the potential for an insect pest management strategy involving the coordinated manipulation of two or more types of digestive enzyme inhibitor genes in plants.

#### INTRODUCTION

Proteinases are essential for insect growth and development, particularly during digestion and molting, and proteinase inhibitors may have detrimental effects on the insect's life cycle when they are present in the diet. Digestive proteinases from many insect species have been studied and, although a diversity of enzymes was found, most were proteins with either a cysteine or serine residue in the active site (Wolfson and Murdock, 1990). Cysteine proteinases provide the major proteolytic activity in the gut of many coleopteran species (Baker, 1982; Gatehouse *et al.*, 1985; Kitch and Murdock, 1986; Murdock *et al.*, 1987; Wieman and Nielsen, 1988; Campos *et al.*, 1989; Thie and Houseman, 1990; Liang *et al.*, 1991; Hines *et al.*, 1991; Silva and Xavier-Filho, 1991; Chen *et al.*, 1992; Gillikin *et al.*, 1992; Purcell *et al.*, 1992), whereas many Lepidoptera have serine proteinases as their major digestive enzymes (Ishaaya *et al.*, 1971;

Miller *et al.*, 1974; Ahmad *et al.*, 1980; Pritchett *et al.*, 1981; Rubenstein and Polson, 1983; Sasaki and Suzuki, 1982; Broadway, 1989; Houseman *et al.*, 1989; Johnson *et al.*, 1990; Lenz *et al.*, 1991; Purcell *et al.*, 1992; Christeller *et al.*, 1992; Lemos and Terra, 1992). A third class of proteinases, acidic proteinases, have been detected in larval midguts of Coleoptera (Silva and Xavier-Filho, 1991) and possibly Diptera (Law *et al.*, 1977; Applebaum, 1985). However, this class of enzymes is not as extensively characterized as are the other two classes.

The effect of a proteinase inhibitor on an insect depends upon several factors, including the spectrum of gut proteinases, the type of inhibitor administered, and the quality of the insect's diet (Broadway and Duffey, 1986a, b, 1988; Burgess *et al.*, 1991; Hinks *et al.*, 1991). When added to diets, serine proteinase inhibitors (SPIs), such as several soybean trypsin inhibitors, potato proteinase inhibitor II, cowpea trypsin inhibitor, and cabbage trypsin and chymo-trypsin inhibitors, retarded the growth of several lepidopteran, coleopteran and dipteran insects including *T. castaneum*, the European corn borer, *Ostrinia nubilalis*, the cowpea weevil, *Callosobruchus maculatus*, the horn fly, *Haematobia irritans*, and the stable fly, *Stomoxys calcitrans* (reviewed in Table 1). In some species these SPIs apparently caused reduced growth due to hypersecretion of digestive enzymes

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 †Abbreviations are: SPI, serine proteinase inhibitor; CPI, cysteine proteinase inhibitor; E-64, L-trans-epoxysuccinyl-leucylamino-(4-guanidino)-butane; LBTI, lima bean trypsin inhibitor; OMTI, ovomucoid trypsin inhibitor; SBBI, soybean Bowman-Birk trypsin inhibitor; STI, soybean Kunitz trypsin inhibitor.

Table 1. Proteinase inhibitors that retard insect growth when administered in diets

Inhib	Proteinase Active Site	Species	Dietary Level*	Reference
Cabbage inhibitors	Serine (trypsin and chymotrypsin)	<i>Trichoplusia ni</i>	Crude extract used	Broadway and Colvin, 1992
		<i>Pieris rapae</i>	Crude extract used	Broadway and Colvin, 1992
Rice cystatin	Cysteine	<i>Tribolium castaneum</i>	10	Chen <i>et al.</i> , 1992
E-64	Cysteine	<i>T. castaneum</i>	0.1-1	Chen <i>et al.</i> , 1992; Oppert <i>et al.</i> , 1993
		<i>Acanthoscelides obiectus</i>	0.01-0.1	Hines <i>et al.</i> , 1990
		<i>Callisobruchus maculatus</i>	0.01-0.25	Murdock <i>et al.</i> , 1988
		<i>Leptinotarsa decemlineata</i>	Applied topically (20 µg/cm <sup>2</sup> )	Wolfson and Murdock, 1987
		<i>T. castaneum</i>	0.1, 1.0, respectively	Oppert <i>et al.</i> , 1993
E-64 plus soybean trypsin inhibitor	Cysteine and serine	<i>T. castaneum</i>	0.1, 1.0, respectively	Oppert <i>et al.</i> , 1993
E-64 plus lima bean trypsin inhibitor	Cysteine and serine	<i>T. castaneum</i>	0.1, 1.0, respectively	Oppert <i>et al.</i> , 1993
Cowpea trypsin inhibitor	Serine (trypsin and chymotrypsin)	<i>C. maculatus</i>	0.1-0.8	Gatehouse <i>et al.</i> , 1979; Gatehouse and Boulter, 1983
		<i>Heliothis virescens</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Heliothis zea</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Spodoptera littoralis</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Chilo partellus</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Anthonomus grandis</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Diabrotica undecimpunctata</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>C. maculatus</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Tribolium confusum</i>	Not given	Hilder <i>et al.</i> , 1990
		<i>Cottylestra zealandica</i>	Not given	Hilder <i>et al.</i> , 1990
Leupeptin	Serine and cysteine	<i>Haemotobia irritans</i>	0.02 (w/v)	Spates and Harris, 1984
		<i>T. castaneum</i>	5.0, 10.0	Birk and Applebaum, 1960; Oppert <i>et al.</i> , 1993
Soybean trypsin inhibitor	Serine	<i>H. zea</i>	0.045-0.18	Broadway and Duffey, 1986b
		<i>Spodoptera exigua</i>	0.045-0.18	Broadway and Duffey, 1986b
		<i>Telogeophyllus commodus</i>	0.33 (w/v)	Burgess <i>et al.</i> , 1991
		<i>H. irritans</i>	0.1 (w/v)	Spates and Harris, 1984
		<i>Stomoxys calcitrans</i>	Not given	DeLoach and Spates, 1980
		<i>Oscinia nubilalis</i>	2.0-5.0	Steffens <i>et al.</i> , 1978
		<i>Manduca sexta</i>	5.0	Shukle and Murdock, 1983
		<i>H. zea</i>	0.045-0.18	Broadway and Duffey, 1986b
		<i>S. exigua</i>	0.045-0.18	Broadway and Duffey, 1986b
		<i>T. commodus</i>	0.33	Burgess <i>et al.</i> , 1991

\*Values are weight percent unless specified otherwise

that led to a deficiency of essential amino acids (Liener, 1979; Broadway and Duffey, 1986b).

Cysteine proteinase inhibitors (CPIs) reduced growth rates and/or increased mortalities for several coleopteran species (Table 1). A recombinant form of a CPI from rice, oryzacystatin (Abe and Arai, 1985), reduced the growth rate of *T. castaneum* larvae when added to the diet at a level of 10% (Chen *et al.*, 1992). E-64 [L-trans-epoxysuccinyl-leucylamino-(4-guanidino)-butane], a CPI from the mold, *Aspergillus japonicus* (Hanada *et al.*, 1978), increased developmental time and mortality for the bean weevil, *Acanthoscelides obiectus* (Hines *et al.*, 1990), *C. maculatus* (Murdock *et al.*, 1988), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Wolfson and Murdock, 1987). Addition of a mixture of amino acids to E-64-treated artificial seeds prevented the effect of the CPI on *A. obiectus* (Hines *et al.*, 1990). In insect diets, both CPIs and SPIs apparently cause amino acid deficiencies that lead to detrimental physiological effects.

Since plant derived proteinaceous inhibitors of either serine or cysteine proteinases retarded or prevented growth and development of several insect species when incorporated into artificial diets, it appears likely that at least some of these inhibitors are a natural mechanism for a plant's defense against insect predation *in vivo* (Birk and Applebaum, 1960; Applebaum and Konijn, 1966; Yeter *et al.*, 1979;

Gatehouse *et al.*, 1986; Broadway and Duffey, 1986b; Hilder *et al.*, 1987; Hines *et al.*, 1990, 1991; Ryan, 1990; Chen *et al.*, 1992; Broadway and Colvin, 1992). Plants, in evolving resistance mechanisms against insects, have probably utilized combinations of different kinds of insect control proteins for defense, including several types of proteinase inhibitors (Ryan, 1983). Soybean, for example, contains both serine and cysteine proteinase inhibitors. A crude extract from soybean meal inhibited *Tribolium* larval growth (Lipke *et al.*, 1954) and was subsequently found to contain a trypsin inhibitor as well as several other uncharacterized inhibitors of *Tribolium* proteolytic activity (Birk *et al.*, 1962).

Cysteine proteinases are predominant enzymes in *T. castaneum* larval gut extracts (Murdock *et al.*, 1987; Chen *et al.*, 1992). Recently, a soybean CPI was partially characterized and found to inhibit the proteolytic activity of *Tribolium* (Hines *et al.*, 1991). Extracts from soybean and other legume seeds were also inhibitory to the cysteine proteolytic activity in gut extracts from *A. obiectus* (Hines *et al.*, 1992).

Data are presented in this paper demonstrating that combinations of a CPI and selected SPIs in the diet are more effective in retarding *T. castaneum* growth and development than are diets containing only a single inhibitor. When added to a wheat germ diet, the combination of E-64 and STI was synergistic in inhibiting *T. castaneum* growth and causing mor-

talities. The detrimental effects were prevented by supplementing the SPI plus CPI treated diets with a mixture of amino acids, suggesting that growth inhibition and toxicity were caused by a deficiency of one or more amino acids. The results indicate that manipulation of digestive enzyme inhibitors in plants for the purpose of conferring host plant resistance to insects may be more effective when more than one class of inhibitor gene is expressed simultaneously.

## MATERIALS AND METHODS

### Biological assays

*T. castaneum* eggs were obtained from a laboratory stock culture reared on a mixture of 95% ground hard red winter wheat flour mixed with 5% torula yeast. Experiments were conducted at 29°C, 60-70% relative humidity, and a 16L:8D photoperiod. Proteinase inhibitors were dissolved in water and blended with wheat germ that had been ground and passed through a 20 mesh wire screen. The diet was frozen, lyophilized, ground in a mortar with a pestle, and equilibrated at 29°C and 60-70% relative humidity.

A single egg was added to 30 mg of diet, and the larval growth rate and mortality determined for 9-10 insects per treatment. At indicated intervals up to the pupal stage, weights of individual larvae were measured using a Cahn microbalance; thereafter, insects were monitored for mortality. The amino acid mixture was that of Taylor and Medici (1966), which contains arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, glutamic acid, glycine and cysteine.

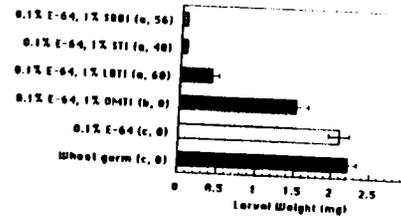


Fig. 1. The effect of a 1% level of various serine proteinase inhibitors combined with 0.1% of the cysteine proteinase inhibitor E-64 in wheat germ on the weight of *T. castaneum* larvae 17 days after hatching. Mean values  $\pm$  SE ( $n = 5-10$ ). Data with the same letter (in parentheses) were not statistically different ( $\alpha = 0.05$ ), as determined by Tukey statistical analysis. Percent mortality for each group is indicated in parentheses following statistical letter. Abbreviations are: SBTI, soybean Bowman Birk trypsin inhibitor; STI, soybean Kunitz trypsin inhibitor; LBTI, lima bean trypsin inhibitor; OMTI, ovomucoid trypsin inhibitor; and E-64, (L-trans-epoxysuccinyl-leucylamino-(4-guanidino)-butane). Larvae fed 1% SBTI, STI, LBTI, and OMTI were approximately the same size as larvae fed only wheat germ (data not shown).

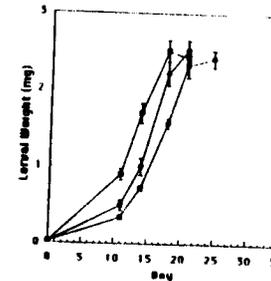


Fig. 2. The effect of 10% STI (●) (a) or 1% E-64 (■) (b) on the growth of *T. castaneum*. Mean values  $\pm$  SE ( $n = 10$ ). Treatments with the same letter (indicated in parentheses following legend identification) were not statistically different ( $\alpha = 0.05$ ), as determined by Tukey statistical analysis of individual regression coefficients of larval weights between days 11 and 18.

### Inhibitors

Kunitz soybean trypsin inhibitor (STI, Type I-S), Bowman Birk soybean trypsin inhibitor (SBBI), lima bean trypsin inhibitor (LBTI, Type II-L), ovomucoid trypsin inhibitor (OMTI, Type III-O) and E-64 were obtained from Sigma Chemical Co.

### Statistical analysis

Statistical tests of significant differences between insect weights and mortalities as a function of diet treatments were analyzed by linear regression, chi-square and Tukey HSD multiple comparison analyses (Tukey, 1951), using the software program SYSTAT (Wilkinson, 1989).

## RESULTS

A survey for the effects on *T. castaneum* growth of several SPIs, added individually or in combination with the CPI, E-64, to wheat germ, revealed a range of effects (Fig. 1). Neither the SPIs at 1% nor E-64 at 0.1%, added separately to diets, significantly reduced larval weight gain 17 days after hatching (only the E-64 diet data are presented; the weight gain of larvae reared on the SPI diets is not significantly different from that of larvae on the control diet). When the SPIs were combined at 1% levels with 0.1% E-64, the Kunitz (STI) and the Bowman Birk (SBBI) trypsin inhibitors from soybean were the most effective proteins that decreased larval growth (97% reduction at 17 days), followed by lima bean trypsin inhibitor (LBTI, 81% reduction) and ovomucoid trypsin inhibitor (OMTI, 31% reduction). Significant mortality occurred with combinations of 0.1% E-64 plus 1% SBTI (56%), LBTI (60%) or STI (40%), and most of those larvae died prior to day 12 when the first observation was made (chi-square analysis,  $\alpha = 0.05$ ). No mortality occurred with the combi-

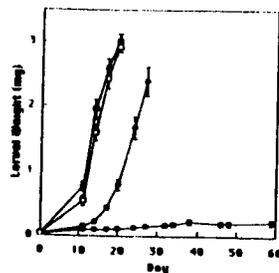


Fig. 3. The effect of 1% STI plus 0.1% E-64 (●) (a) in wheat germ compared to wheat germ alone (■) (b), 7% amino acid mixture added to wheat germ (□) (c), or 7% amino acid mixture plus the inhibitors (1% STI + 0.1% E-64) (○) (d) added to wheat germ on the growth of *T. castaneum*. Means values  $\pm$  SE ( $n = 10$ ). Treatments with the same letter (indicated in parentheses following legend identification) were not statistically different ( $\alpha = 0.05$ ), as determined by Tukey statistical analysis of individual regression coefficients of larval weights between days 11 and 27.

nation of 1% OMTI plus 0.1% E-64 or in the treatment with 0.1% E-64 alone.

The effect of 1% E-64 or 10% STI, added individually to the diet, on the weight of *T. castaneum* larvae during the first 25 days of development is shown in Fig. 2. Both E-64 and STI reduced larval weight gain until day 21, at which time all groups of larvae weighed about the same. Maximal weight (2.2–2.7 mg) was obtained by approximately 18 days in both the control and 10% STI groups, whereas the 1% E-64 group attained the same weight about three days later. The former two groups of larvae began to pupate by day 21 and the latter group after about day 25. Both the 10% STI- and 1% E-64-supplemented diets produced mortalities of only 10%.

Since a mixture of either of the two soybean trypsin inhibitors with E-64 was effective in retarding *T. castaneum* growth (Fig. 1), the combination of E-64 and STI was selected for further study. When added at levels lower than were effective individually, 0.1% E-64 plus 1% STI dramatically reduced larval weight gain (Fig. 3). One of the larvae died before day 11 and, although nine out of ten insects reared on the proteinase inhibitor combination diet survived for several weeks, all remaining larvae exhibited little weight gain and eventually died before pupation. However, when the STI plus E-64 inhibitor-treated diet was supplemented at 7% with a mixture of amino acids, larval growth was restored, although at a somewhat slower overall rate than that of control larvae. Little or no mortality ( $\leq 10\%$ ) occurred with larvae fed either control, amino acid mixture-supplemented, or amino acid mixture plus inhibitor mixture-supplemented diets.

Flour beetle larvae that were fed 0.1% E-64 plus either 5 (data not shown) or 10% STI-treated wheat

germ were severely affected and died during the first or second instar (Table 2). When the 0.1% E-64 plus 10% STI diet was supplemented at 20% with the amino acid mixture, the effect of the inhibitor mixture was subdued. Although the larvae grew slowly at first, 89% of the test group eventually developed into adults.

#### DISCUSSION

E-64 and STI added individually to wheat germ at relatively high concentrations (1% and 10%, respectively) slowed *T. castaneum* growth, but caused little or no mortality. During the first few weeks, larvae reared on wheat germ containing 10% STI were approximately 50% smaller than larvae reared on wheat germ alone, and larvae fed 1% E-64 supplemented diets were even smaller. However, nearly all of these larvae eventually grew to their fully matured size, pupated, and eclosed to adults.

When combinations of SPIs and E-64 were fed to *Tribolium* larvae, a synergistic effect on growth retardation and mortality was observed. Results of an SPI feeding survey for effects on *T. castaneum* indicated that, when administered together with E-64, the two soybean trypsin inhibitors were the most effective in reducing *T. castaneum* larval growth. LBTI was less effective than STI or SBBI in retarding larval growth, but it also caused high mortality. OMTI was the least effective proteinaceous inhibitor tested in combination.

When relatively low concentrations of two inhibitors (0.1% E-64 and 1% STI) were added in combination to a wheat germ diet, flour beetle larval growth was severely retarded. The synergism exhibited resulted in greater growth inhibition and mortality than was predicted from bioassays incorporating individual inhibitors. Although 100% mortality was observed for larvae fed the 0.1% E-64 plus 1% STI-treated diet, supplementation of this diet with a 7% amino acid mixture prevented nearly all of the premature mortality.

Combining 0.1% E-64 and a relatively high level of STI (10%) in the diet resulted in 100% mortality in the first or second larval instar. Supplementation of this diet with a 20% amino acid mixture partially restored growth and resulted in only 11% mortality. Hines *et al.* (1990) found that addition of an amino

Table 2. The effect of 0.1% E-64 plus 10% STI on the growth of *Tribolium castaneum* larvae\*

Treatment	Larval weight†	Mortality (%)
None	1.41 $\pm$ 0.6 (9)	0
0.1% E-64 + 10% STI	0.08 (1)	100
0.1% E-64 + 10% STI + 20% amino acid mixture	0.25 $\pm$ 0.04 (8)	11
20% amino acid mixture	0.96 $\pm$ 0.10 (9)	0

\*Proteinase inhibitors were added as indicated to a diet consisting of 80% wheat germ and 20% white flour. Amino acid mixture (Taylor and Medici, 1966) was added at 20% to the indicated treatments.

†Weight in mg, determined 12 days after hatching. Data are the mean  $\pm$  SE. Number of larvae weighed given in parentheses.

acid mixture to an E-64 treated diet did not prevent inhibition of gut proteolytic enzyme activity of *A. obiectus*, but did result in reversal of both delayed development and high mortality. Similarly, Broadway and Duffey (1986b) demonstrated that the presence of serine proteinase inhibitors in the diets of the corn earworm, *Heliothis zea*, and fall armyworm, *Spodoptera exigua*, resulted in an increased level of tryptic activity. Since methionine was able to overcome the effect of serine proteinase inhibitors on *H. zea* and *S. exigua*, these authors concluded that growth inhibition was due to a limiting amount of sulfur containing amino acids. We did not attempt to rescue *T. castaneum* from the growth inhibiting effects of proteinase inhibitor combinations using only methionine instead of the amino acid mixture. However, when a 1% E-64-treated diet was supplemented with either 0.5% methionine or tryptophan, the growth rate was approximately the same as that of the control group (data not shown), indicating that the CPI was causing a deficiency of those amino acids. Our results are not directly comparable to those of Broadway and Duffey (1986b), who tested two species of moths instead of a beetle. However, the mechanism by which proteinase inhibitors slow growth and cause mortality in all three of these species is probably similar and involves a deficiency in one or more amino acids essential for development.

Although CPIs, such as E-64 and soybean or rice cystatin, can inhibit 80% or more of the proteolytic activity of gut extracts from *Tribolium* (Murdock *et al.*, 1987; Hines *et al.*, 1991; Chen *et al.*, 1992), we found that the 1% E-64-treated diet had little or no effect on *Tribolium* viability, even though it did reduce larval weight gain in the early weeks following hatching. Previous studies revealed only slight effects of SPIs on the growth of *T. castaneum* (Birk and Applebaum, 1960), and our results using 1–10% SPIs are in agreement. Although some studies failed to detect inhibition of the proteolytic activity of *T. castaneum* or *T. confusum* by SPIs *in vitro* (Birk and Applebaum, 1960; Lipke *et al.*, 1954), more recent evidence suggests that serine proteinases account for 14 to 36% of the proteolytic activity in midgut extracts from *T. castaneum* (Murdock *et al.*, 1987; Chen *et al.*, 1992). In view of our results on the toxicity of multiple inhibitors, insect gut extracts should be assayed for the effects of inhibitor combinations as well as single inhibitors. Whereas some SPIs or CPIs individually may have little effect on midgut proteolytic activity *in vitro*, combinations may exhibit a synergism similar to that we have demonstrated in feeding assays. The relative importance of both cysteine and serine proteinases in the digestion of food by *T. castaneum* may account for the synergistic effects on larval growth and mortality observed with dietary mixtures of CPIs and SPIs.

Single inhibitors with separate binding sites for different types of digestive enzymes may be more effective as insect resistance factors than are those

that bind exclusively to only one type of enzyme. As was observed for potato proteinase inhibitor II, which inhibits both trypsin-like and chymotrypsin-like enzymes, in bioassays of the black field cricket, *T. commodus* (Burgess *et al.*, 1991). Similarly, utilization of mixtures of inhibitors that together target more than one digestive enzyme class may exhibit increased toxicity toward insects. Artificial diets containing extracts of young foliage of mature cabbage plants, which contain high levels of both chymotrypsin inhibitors and amylase inhibitors, retarded the growth and development of the cabbage looper, *Trichoplusia ni*, and the cabbage butterfly, *Pieris rapae* (Broadway and Colvin, 1992). Levels of amylase inhibitors and trypsin inhibitors coexpressed in cowpea were also correlated to bruchid resistance (Piergiovanni *et al.*, 1991).

A mixture of inhibitors selective for all classes of proteinases involved in an insect's digestion would be the best combination of inhibitors to use for diminishing insect growth. Synergism between inhibitors resulting in insect growth inhibition may allow the application of very small amounts of inhibitors in plants for host plant resistance, and these low levels would lessen concerns about side effects of digestive proteinase inhibitors added to vertebrate food sources. Since other animal digestive systems may also be adversely affected by proteinase inhibitors used as insect control proteins, only inhibitors that are selective for insect enzymes and/or degraded by pepsin under the acidic conditions of the vertebrate stomach should be developed for insect control. This selectivity or instability would help to minimize side effects that may be encountered when inhibitors are incorporated into vertebrate food sources. CPIs are preferred candidates in this regard due to the utilization by vertebrates of acidic and serine proteinases instead of cysteine proteinases for digestion.

Results obtained from feeding studies and *in vitro* enzyme activity assays employing proteinase inhibitors have illustrated the potential use of these inhibitors as antinutritional factors for insects. Table 1 lists the species that have exhibited reduced growth rates and/or increased mortalities when fed proteinase inhibitors. Many species of Coleoptera, Lepidoptera, Diptera and Orthoptera are susceptible to CPIs and/or SPIs when administered in the diet. This information is useful when choosing one or more candidate proteinase inhibitors for development as insect control proteins (Hilder *et al.*, 1990; Wolfson, 1991). According to our knowledge, no testing of mixtures of enzyme inhibitors in insect feeding studies has been reported previously.

Proteinase inhibitor genes have been expressed in potato (Hendriks *et al.*, 1991), tobacco (Sanchez-Serrano *et al.*, 1987; Hilder *et al.*, 1989), and tomato (Hilder *et al.*, 1987), and all of the transgenic plants exhibited increased resistance to certain insect pests. A serine proteinase inhibitor gene fused to a truncated *Bacillus thuringiensis* endotoxin gene was found

the efficacy of the insecticidal endotoxin in transgenic tobacco (MacIntosh *et al.*, 1990). Proteinase inhibitors that bind multiple types of gut endopeptidases were more effective in reducing the growth of *Teleogryllus commodus* than inhibitors that bind only one type of enzyme (Burgess *et al.*, 1991). Transgenic tobacco expressing tomato inhibitor II (a protein that inhibits both trypsin and chymotrypsin) retarded the growth of *M. sexta* larvae, whereas transgenic tobacco expressing tomato inhibitor I (a protein that inhibits only chymotrypsin) was more susceptible to larval damage (Johnson *et al.*, 1989). Manipulation of combinations of genes that code for proteins inhibitory to insect digestive enzymes should be more effective as a control strategy than the manipulation of only a single inhibitor gene in plants. The expression of multiple enzyme inhibitor genes in transgenic plants may have the additional advantage of a reduced risk of pest adaptation.

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