

## Effects of bean and wheat $\alpha$ -amylase inhibitors on $\alpha$ -amylase activity and growth of stored product insect pests\*

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Accepted: October 6, 1994

**Key words:** amylase inhibitor, red kidney bean, hard red winter wheat, growth, insects, beetles, plant resistance, stored products, protease inhibitor

### Abstract

Insect  $\alpha$ -amylase inhibiting and/or growth inhibiting activities of proteinaceous inhibitors from red kidney bean (*Phaseolus vulgaris*) and hard red winter wheat (*Triticum aestivum*) were examined. The bean inhibitor was most effective *in vitro* against  $\alpha$ -amylases from the red flour beetle (*Tribolium castaneum*) and the confused flour beetle (*T. confusum*), followed by those from the rice weevil (*Sitophilus oryzae*) and yellow mealworm (*Tenebrio molitor*). The insect enzymes were from two- to 50-fold more susceptible than human salivary  $\alpha$ -amylase. When the inhibitors were added at a 1% level to a wheat flour plus germ diet, the growth of red flour beetle larvae was slowed relative to that of the control group of larvae, with the bean inhibitor being more effective than the wheat inhibitor. Development of both the red flour beetle and flat grain beetle (*Cryptolestes pusillus*) was delayed by 1% bean inhibitor, but development of the sawtoothed grain beetle (*Oryzaephilus surinamensis*) and lesser grain borer (*Rhyzopertha dominica*) was not affected by either the bean or wheat inhibitor at the 1% level. Rice weevil adults fed a diet containing 1% bean or wheat inhibitor exhibited more mortality than weevils fed the control diet. When the wheat amylase inhibitor was combined with a cysteine protease inhibitor, E-64, and fed to red flour beetle larvae, a reduction in the growth rate and an increase in the time required for adult eclosion occurred relative to larvae fed either of the inhibitors separately. The bean inhibitor was just as effective alone as when it was combined with the protease inhibitor. These results demonstrate that plant inhibitors of insect digestive enzymes act as growth inhibitors of insects and possibly as plant defense proteins, and open the way to the use of the genes of these inhibitors for genetically improving the resistance of cereals to storage pests.

### Introduction

Seeds contain a number of plant proteins that are toxic to mammals and insects. Some of these, such as ribo-

some inactivating proteins, are extremely toxic, whereas others, such as the inhibitors of digestive enzymes, are much less toxic and primarily exert their action by slowing down the digestion of the seed material ingested by a predator (García-Olmedo *et al.*, 1987). Because insects and their food sources have evolved together, proteins of a plant species are usually not effective against insects that attack that species or products derived therefrom. However, if the genes that encode such proteins could be transferred from one plant species to another, this could protect the

\* Cooperative investigation between the Agricultural Research Service, the University of California, San Diego, and the Kansas Agricultural Experiment Station (Contribution no. 94-416-J). Supported in part by a grant from the Ministry of Education and Science, Spain-Fulbright Program to J. J. P. Mention of a proprietary product does not constitute a recommendation or endorsement by the USDA. The USDA is an equal opportunity/affirmative action employer and all agency services are available without discrimination.

transformed plant against its otherwise successful herbivores.

The  $\alpha$ -amylase inhibitor of the common bean (red kidney bean), *Phaseolus vulgaris*, inhibits  $\alpha$ -amylases from mammals, as well as several species of bruchid insects that are storage pests of legume seeds (Ishimoto & Kitamura, 1989). Furthermore, in an artificial seed assay, this protein inhibited the development of larvae of bruchid species that normally attack cowpeas, mungbeans, and chickpeas (Ishimoto & Kitamura, 1989; Huesing *et al.*, 1991). This amylase inhibitor did not inhibit  $\alpha$ -amylases from bruchids that attack the common bean, allowing successful predation of that legume by the Mexican bean weevil, *Zabrotes subfasciatus*, and the bean weevil, *Acanthoscelides obtectus*. However, a wild bean-derived inhibitor has been described recently, which suppresses growth of *Z. subfasciatus* when administered in an artificial diet at a level of 1% (Suzuki *et al.*, 1993). Recently, we isolated the gene for the  $\alpha$ -amylase inhibitor of *P. vulgaris* (Moreno & Chrispeels, 1989). We also isolated several amylase inhibitors from hard red winter wheat, *Triticum aestivum*, and determined their inhibitory selectivities for some insect and mammalian  $\alpha$ -amylases (Feng *et al.*, 1991a). One of these amylase inhibitors, which eluted during reversed-phase high performance liquid chromatography as peak fraction number 24 and was identified as a 0.19 inhibitor (Feng, 1990), inhibited  $\alpha$ -amylases from the rice weevil, *Sitophilus oryzae*; confused flour beetle, *Tribolium confusum*; and human saliva. However, this inhibitor did not inhibit porcine pancreatic  $\alpha$ -amylase. Many of these inhibitors appear to be relatively selective towards insect  $\alpha$ -amylases and have the capability of providing plants with resistance to insect pests.

Using recombinant DNA technology and plant transformation, we are now exploring the possibility of transferring the inhibitor genes from the kidney bean and hard red winter wheat to other plants such as rice and maize with the goal of obtaining plants that are resistant to insect pests. Because grains such as wheat, rice, and maize are some of the world's most important crops and also are attacked by many storage pests, and because some of these plants can now be transformed with enzyme inhibitor genes, we determined the effects of the bean amylase inhibitor on  $\alpha$ -amylases from several insect pests of cereal grains *in vitro*. We also examined the effects of bean and wheat amylase inhibitors, a protease inhibitor, and combinations thereof on the growth of several stored product insect species in feeding assays.

## Materials and methods

**Inhibitor preparation.** The procedure used to purify the amylase inhibitor from the red kidney bean is a combination of previous protocols (Marshall & Lauda, 1975; Powers & Whitaker, 1977; Pick & Wöber, 1978). Two hundred grams of beans were ground in a coffee mill. The flour was extracted overnight in 1.2 L of 10 mM  $\beta$ -mercaptoethanol at room temperature, and the suspension centrifuged at  $13\,000 \times g$  for 60 min at 4 °C. The pellet was discarded and the supernatant adjusted to pH 4.0 with HCl. The new suspension was heated at 65 °C for 30 min and centrifuged at  $13\,000 \times g$  for 60 min at 4 °C. The pellet was discarded, and the supernatant adjusted to pH 7.0 with  $\text{Na}_2\text{HPO}_4$ . Protein precipitated by ammonium sulfate between 40% and 65% saturation contained most of the inhibitor. The pellet was resuspended in 20 mM sodium phosphate buffer (pH 7.6) containing 50 mM NaCl, loaded onto a pre-equilibrated DEAE-cellulose column (45  $\times$  3 cm), washed with the same buffer, and eluted with a NaCl gradient (50–250 mM) in buffer. Fractions were collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, and those containing the pure amylase inhibitor were pooled. In order to remove any possible traces of phytohemagglutinin, the pooled sample was shaken with 10 mL of thyroglobulin-Sepharose 4B beads for 2 h at room temperature and filtered through a porous glass disk. The filtrate was dialyzed against water and lyophilized.

The wheat amylase inhibitor was purified from hard red winter wheat (cv Newton) kernels as described by Feng *et al.* (1991a) and corresponded to HPLC peak number 24 of Fig. 3 in that study. It is a member of the 0.19 subfamily of wheat inhibitors (Feng, 1990), which is a group of dimeric inhibitors of both insect and mammalian  $\alpha$ -amylases (Silano, 1987).

The cysteine proteinase inhibitor, E-64 [L-*trans*-epoxysuccinyl-leucylamino-(4-guanidino)-butane], was obtained from Sigma Chemical Co. (St. Louis, MO).

**Electrophoresis and immunoblotting.** SDS-PAGE using 15% acrylamide was performed according to Laemmli (1970). After transfer to a nitrocellulose membrane, proteins were detected using a rabbit antibeane amylase inhibitor serum obtained as described by Moreno & Chrispeels (1989). We used goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad Labs., Hercules, CA) as the secondary antibody. Proteins were visualized by staining with Coomassie bril-

liant blue.

**Insects.** Insects were obtained from stock cultures at the U.S. Grain Marking Research Laboratory. *Tribolium castaneum* (red flour beetle), *T. confusum* (confused flour beetle), and *Oryzaephilus surinamensis* (saw-toothed grain beetle) were maintained on a mixture of 95% hard red winter wheat flour mixed with 5% torula yeast. *Sitophilus oryzae* (rice weevil) and *Rhyzopertha dominica* (lesser grain borer) were reared on whole wheat. *Tenebrio molitor* (yellow mealworm) was reared on 80% wheat flour, 15% rolled oats, and 5% yeast, and *Cryptolestes pusillus* (flat grain beetle) on 45% wheat flour, 50% rolled oats, and 5% yeast.

**Amylase purification and assay.**  $\alpha$ -Amylases from adult insects were isolated and assayed using 1% potato starch as substrate according to Chen *et al.* (1992a). Human salivary  $\alpha$ -amylase (type IX-A) was obtained from Sigma Chemical Co. (St. Louis, MO). Inhibitor concentrations that gave 50% inhibition ( $IC_{50}$ ) were derived from plots of percent inhibition versus inhibitor concentration, which varied from 10 to 450 nM.

**Bioassays.** Bioassays were conducted at approximately 30 °C and 70% relative humidity with a 16L:8D photoperiod. The amylase inhibitor was mixed with either wheat germ, a wheat flour/germ diet I (20% hard red winter wheat flour plus 80% ground wheat germ) or a wheat flour/germ diet II (80% hard red winter wheat flour plus 20% ground wheat germ). Diets were mixed with 1.5 volumes of water, freeze-dried, and then ground into a powder in a mortar with a pestle before equilibrating at 30 °C and 70% humidity for several days. Diets for the lesser grain borer were compacted to allow easier locomotion by the grub-like larvae. Insect eggs that had been oviposited within a 20 h period or adults approximately 1 day old were placed in individual containers with the diet. Red flour beetle larvae were weighed 11 and 14 days after egg hatch and lesser grain borer larvae after 19 days. Mortality and time of adult eclosion also were recorded.

Bioassays were not performed with rice weevil larvae, because of procedural difficulties in handling the eggs and larvae that normally grow and develop within grain kernels. Because prior work has shown that adult weight gain and longevity of the rice weevil and its sibling species, *S. zeamais*, are dependent on dietary starch and not protein (Chippendale, 1972; Brown & Chippendale, 1975), the adult rice weevil was used for

bioassay. Each weevil was weighed at 0–24 h after adult eclosion, placed on 10 mg of diet, and then weighed again 7 days later.

**Data analysis.** Statistical tests of significant differences between diet treatments included the Tukey HSD multiple comparison test, independent t-test or Fisher exact test using the software program SYSTAT (Wilkinson, 1989).

## Results

**Amylase inhibitor purification.** SDS-PAGE and Coomassie brilliant blue staining of the purified bean  $\alpha$ -amylase inhibitor (Fig. 1A) showed five polypeptides ranging in apparent molecular weight from 14 to 19 kDa, together with some faint bands around 30 kDa, which have been shown to be aggregates of the smaller polypeptides (Moreno & Chrispeels, 1989; Moreno *et al.*, 1990). This banding pattern was identical to that of the pure protein preparation obtained by affinity adsorption to porcine pancreatic  $\alpha$ -amylase coupled to agarose beads (Moreno & Chrispeels, 1989; Moreno *et al.*, 1990) and corresponded exactly with the banding pattern obtained by immunoblotting with a polyclonal rabbit serum against the  $\alpha$ -amylase inhibitor (Fig. 1B).

The wheat  $\alpha$ -amylase inhibitor exhibited a single Coomassie brilliant blue-stained band after non-denaturing PAGE, a staining pattern identical to that obtained by Feng *et al.* (1991a).

**Effect of amylase inhibitors on insect and human  $\alpha$ -amylases in vitro.** Amylase inhibitor activity was tested *in vitro* using enzymes from *T. castaneum*, *T. confusum*, *S. oryzae*, and *T. molitor*, and also human salivary  $\alpha$ -amylase. From data reported previously (Feng *et al.*, 1991a), we estimated that the  $IC_{50}$  values for inhibition of *S. oryzae*, *T. molitor* and human salivary  $\alpha$ -amylases by the wheat amylase inhibitor were  $\leq 0.3 \mu\text{M}$ . These values were in the same order of magnitude as the  $IC_{50}$  values for the red kidney bean amylase inhibitor, which ranged from 0.01 to  $>0.50 \mu\text{M}$  (Table 1). The insect enzymes were from two- to 50-fold more susceptible to the inhibitors than the human enzyme. Amylases from the two *Tribolium* species were the most susceptible, followed by the *S. oryzae* and *T. molitor* enzymes.

**Effect of amylase inhibitors on insect growth in vivo.** Because the red flour beetle  $\alpha$ -amylase was very sus-

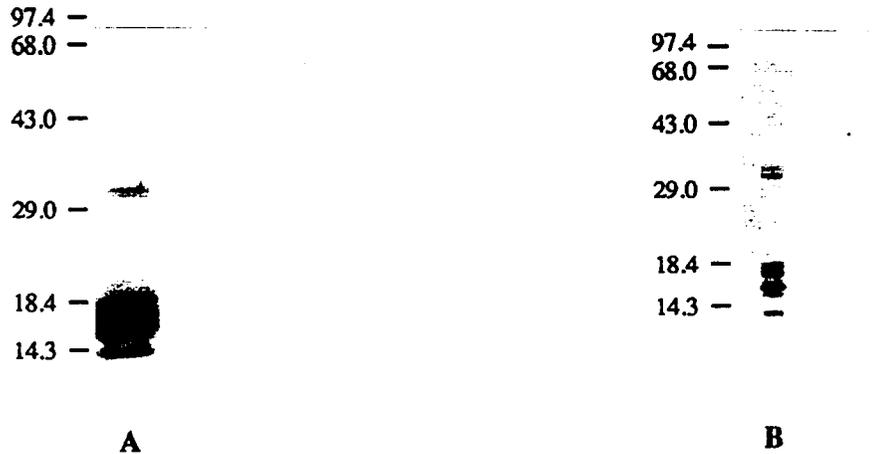


Fig. 1. SDS-PAGE of purified red kidney bean  $\alpha$ -amylase inhibitor. (A) Coomassie brilliant blue protein stain of gel. (B) Immunoblot with rabbit anti-bean  $\alpha$ -amylase inhibitor antiserum.

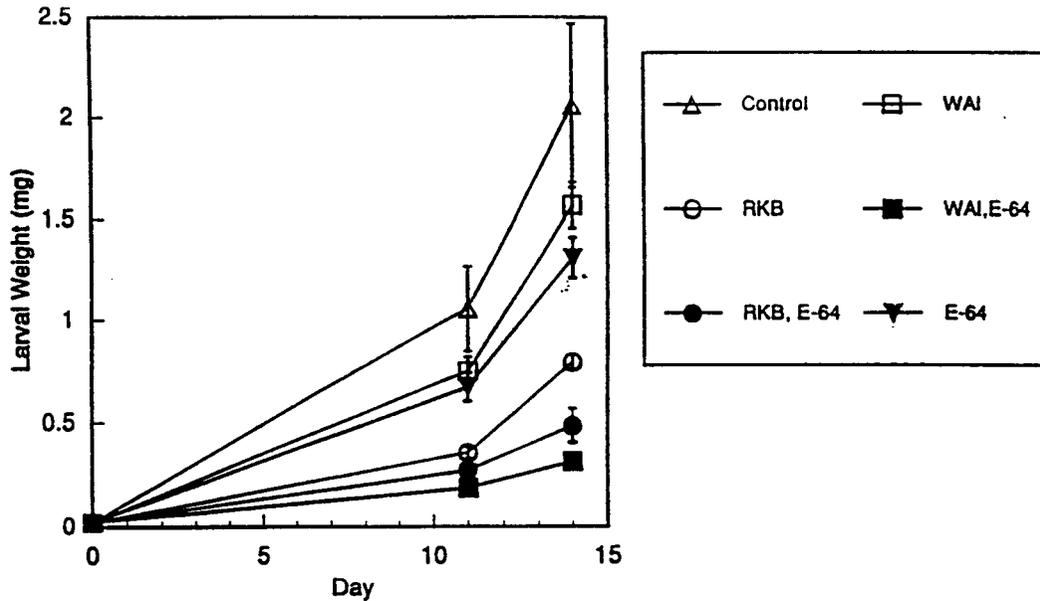


Fig. 2. Effect of red kidney bean and wheat  $\alpha$ -amylase inhibitors with and without E-64 added to wheat flour plus germ diet I on the growth of the red flour beetle, *Tribolium castaneum*. Control diet = open triangles, 1% red kidney bean amylase inhibitor diet = open circles, 1% wheat amylase inhibitor diet = open squares, 0.1% E-64 diet = closed triangles, 1% red kidney bean amylase inhibitor plus 0.1% E-64 diet = closed circles, and 1% wheat amylase inhibitor plus 0.1% E-64 = closed squares (n = 6-10). See Table 2 for statistical analysis of data for day 14.

ceptible to the inhibitors *in vitro*, that species was used primarily in bioassays to test for effects on growth and development. The wheat flour plus germ diet I (see Materials and methods section for diet compositions) with bean amylase inhibitor added at a 1% level slowed the growth rate of neonate larvae relative to that of the control group of larvae (Fig. 2). After 2 weeks, larvae fed bean amylase inhibitor were approximately one-half the size of those fed wheat amylase inhibitor

and one-third the size of control larvae. The weight of larvae fed the wheat inhibitor was not significantly different from that of control larvae. After approximately 4 weeks, significantly fewer insects were in the adult stage on the diet containing bean amylase inhibitor than on either the wheat-inhibitor or the control diet (Table 2). However, all of the larvae fed the amylase inhibitor pupated and eclosed to adults by 6 weeks and there was no difference in mortality between them and

Table 1. Concentration of red kidney bean amylase inhibitor causing 50% inhibition of insect gut and human salivary amylases<sup>1</sup>

Amylase source	IC <sub>50</sub> (μM)
Red flour beetle	0.01
Confused flour beetle	0.02
Rice weevil	0.12
Yellow mealworm	0.23
Human salivary	>0.50

<sup>1</sup> 1% potato starch digested in 20 mM sodium phosphate, 7 mM NaCl, pH 6 (Feng *et al.*, 1991a).

the control insects. The results indicated that the bean amylase inhibitor adversely affected *Tribolium* larval growth and development when it was present at a concentration of 1% in the diet.

Rice weevil  $\alpha$ -amylase was strongly inhibited *in vitro* by the bean amylase inhibitor. To determine whether the bean inhibitor was active *in vivo*, bioassays in which the adult weevil was fed the bean inhibitor mixed with diet II were performed. There was no significant difference in the weights of adults fed 7 days on the control diet or on the inhibitor supplemented diets (Table 3). However, there was an increase in adult mortality after 35 days on diet II containing 1% of either amylase inhibitor.

Other species were also bioassayed with the amylase inhibitors. Development was delayed by approximately 17 days when flat grain beetle larvae were fed milled wheat germ mixed with 1% bean amylase inhibitor (Table 4). Two other coleopteran species, the lesser grain borer and sawtoothed grain beetle, were not affected adversely by either the bean or wheat amylase inhibitor when incorporated at a 1% level in the wheat flour plus germ diet II. No significant difference occurred between treated and untreated *R. dominica* in larval weight (range of means = 1.57–1.72 mg) 19 days after egg hatch or in the time (24.9–26.8 days) between egg hatch and adult eclosion. For *O. surinamensis*, there was no significant difference between treatments in the time (20.2–24.5 days) between egg hatch and adult eclosion.

*Effect of a mixture of amylase inhibitors and a protease inhibitor on T. castaneum.* Digestive enzymes in the midgut of red flour beetle larvae include not only  $\alpha$ -

amylases, but also cysteine and serine proteases (Chen *et al.*, 1992b; Oppert *et al.*, 1993). Individually, the wheat amylase inhibitor (1%) and the cysteine protease inhibitor, E-64 (0.1%), were not particularly effective (Table 2). However, together they acted additively or possibly synergistically, causing a weight gain reduction of 84%. The bean amylase inhibitor, on the other hand, was quite effective by itself (62% reduction in weight gain), but no statistically significant additive effect occurred when it was combined with E-64, possibly because it was already quite effective. Adult eclosion was delayed by the bean inhibitor alone and also by the inhibitor mixture, as shown by the eclosion percentages at 28 days listed in Table 2. However, all of the insects eclosed to adults within 6 weeks after egg hatch.

## Discussion

In insects,  $\alpha$ -amylases are important catabolic enzymes that are needed for the digestion of dietary starch, and  $\alpha$ -amylase inhibitors present in a food source may have detrimental effects on the insect's life cycle. We are interested in the properties of plant-derived proteinaceous inhibitors of insect  $\alpha$ -amylases and their possible manipulation by genetic engineering methods for pest control purposes. Previously, we determined the relative effectiveness of  $\alpha$ -amylase inhibitors from wheat, rice, and maize *in vitro* against  $\alpha$ -amylases from several species of stored product insects (Feng *et al.*, 1991a, b; Chen *et al.*, 1992a). Wheat contains a large number of relatively abundant amylase inhibitors that selectively inhibit insect  $\alpha$ -amylases, whereas rice and maize have lower levels of and relatively fewer inhibitors. In this study, we compared the enzyme-inhibiting and growth-inhibiting activities of  $\alpha$ -amylase inhibitors from bean and wheat. We used the red flour beetle as the primary test insect and demonstrated that the bean amylase inhibitor suppressed larval growth more effectively than the wheat amylase inhibitor. We also combined the amylase inhibitors with a cysteine protease inhibitor and observed that mixtures could be more inhibitory than single inhibitors. Previously, we reported that combinations of a cysteine proteinase inhibitor and serine proteinase inhibitor were toxic to red flour beetle larvae when fed at levels where individual inhibitors were nontoxic (Oppert *et al.*, 1993). Although the wheat amylase inhibitor plus protease inhibitor mixture did

Table 2. Effect of 1% kidney bean and wheat  $\alpha$ -amylase inhibitors with and without the addition of 0.1% protease inhibitor, E-64, on the growth and development of the red flour beetle, *Tribolium castaneum*<sup>1</sup>

Diet	Weight (mg) <sup>2</sup>	% Reduction	Adult eclosion (%) <sup>3</sup>
Control	2.06 $\pm$ 0.41 a	0	88
Wheat amylase inhibitor	1.57 $\pm$ 0.11 ab	20	100
Bean amylase inhibitor	0.79 $\pm$ 0.04 bc	62	20 *
E-64	1.31 $\pm$ 0.10 b	36	100
Wheat amylase inhibitor + E-64	0.32 $\pm$ 0.03 c	84	0 *
Bean amylase inhibitor + E-64	0.49 $\pm$ 0.08 c	76	14 *

<sup>1</sup> Eggs were placed on the wheat flour plus germ diet I (30 mg per insect).

<sup>2</sup> Larval weight 14 days after egg hatch. Mean values  $\pm$  S. E. (n = 6-10). Data with the same letter were not significantly different ( $\alpha = 0.05$ ) as determined by Tukey statistical analysis.

<sup>3</sup> Percentage that were adults (n = 6-10) 29 days after egg hatch. Data marked by an asterisk were significantly different from the control at the  $\alpha = 0.05$  level as determined by Fisher exact test (two-tail).

Table 3. Effect of amylase inhibitors on adult weight and mortality of the rice weevil, *Sitophilus oryzae*, using a wheat flour plus germ diet<sup>1</sup>

Diet	Weight Change (%) <sup>2</sup>	Mortality (%) <sup>3</sup>
Control	1.6 $\pm$ 0.7 a	33
Bean amylase inhibitor		
0.1%	2.7 $\pm$ 0.7 a	50
1.0%	2.8 $\pm$ 0.4 a	80 *
Wheat amylase inhibitor		
0.1%	5.0 $\pm$ 0.7 a	50
1.0%	0.4 $\pm$ 3.9 a	80 *

<sup>1</sup> Each rice weevil adult was placed on 10 mg of wheat germ/flour diet II + amylase inhibitor at 0-24 h after eclosion.

<sup>2</sup> Change after 7 days. Mean values  $\pm$  S.E. (n = 10-15). Values marked with the same letter were not significantly different from the control at the  $\alpha = 0.05$  level as determined by Tukey analysis.

<sup>3</sup> Mortality after 35 days. Values marked by an asterisk were significantly different from the control at the  $\alpha = 0.05$  level as determined by the Fisher exact test (two-tail).

not increase mortality, it did reduce the growth rate of larvae and delayed adult eclosion.

Additional work will be required to determine the cause of premature mortality for weevils in the 1% inhibitor treatment. It is possible that insects on the inhibitor-treated diet may have digested starch less efficiently and consumed relatively more diet in order to meet their energy requirements. These insects may have consumed all of the food that was uncontaminated with feces. The wheat flour/germ diet II contained

about 5% sugar (Pomeranz, 1988), which could be an energy source if digestion of starch was blocked.

It would have been ideal to perform all of the bioassays using the same diet, and, if possible, a diet as close as possible to the insect's natural diet. Although many of the stored product pests feed mainly on the germ, internal feeders such as the rice weevil and lesser grain borer subsist primarily on the endosperm. The tissue preferences of most species have not been quantified, but the confused flour beetle apparently prefers a diet composed of about four-fifths germ and one-fifth

Table 4. Effect of red kidney bean amylase inhibitor on the development of the flat grain beetle, *Cryptolestes pusillus*<sup>1</sup>

Diet	Developmental time (days) <sup>2</sup>	Mortality (%) <sup>3</sup>
Control	29.6 ± 0.5	0
1% Bean amylase inhibitor	46.6 ± 2.0	15

<sup>1</sup> Eggs were placed on the wheat germ diet (2 mg per egg).

<sup>2</sup> Developmental time from egg hatch to adult eclosion. Mean values ± S. E. (n = 11-14). The values were significantly different at the  $\alpha = 0.01$  level as determined by the independent t-test.

<sup>3</sup> Mortality during the larval, pupal and pharate adult stages. Values were not significantly different at the  $\alpha = 0.05$  level as determined by the Fisher exact test.

endosperm based on tests with milled wheat fractions (Waldbauer & Bhattacharya, 1973). Pure germ has no starch, but milled germ contains about 21% starch due to contamination by the endosperm, which contains about 80% starch on a dry weight basis (Pomeranz, 1988). Some evidence indicates that the endogenous levels of amylase inhibitors in the endosperm decrease the amylolytic activity of adult rice weevils (Baker, 1988).

The selectivity of the inhibitor for the insect enzymes may be more important than the choice of diet that is used for the bioassay. The bean amylase inhibitor decreased the developmental rate of the flat grain beetle (on a diet of wheat germ) and the red flour beetle (on a diet of 80% germ plus 20% flour), despite the presence of about 17% sugar in the germ (Pomeranz, 1988), which may tend to rescue insects from the detrimental effects of amylase inhibitors. The bean amylase inhibitor did not inhibit the development of the sawtoothed grain beetle or lesser grain borer, or reduce the adult weight gain of the rice weevil on a diet of 20% germ plus 80% flour. Because the amylase of the red flour beetle was about 10-fold more sensitive to the bean inhibitor than the amylase of the rice weevil *in vitro*, it was perhaps not surprising that one of the most dramatic results of the bioassays of the bean inhibitor occurred with the former insect. The inhibitor delayed development, but caused no mortality with this species. Larvae of the confused flour beetle, a close relative of the red flour beetle, have been shown to utilize dietary protein so effectively that they were able to complete their development even when dietary carbohydrate was virtually absent (Fraenkel & Blewett, 1943).

To be useful as potential plant protection agents, proteins need not be toxic or even inhibitory at low levels. The amylase inhibitors were not very toxic, and their primary effect was a reduction in the rate of insect growth and development. Indeed, slowing down the developmental time and increasing the days to eclosion by 2-fold is sufficient to dramatically reduce the damage done by storage pests when the storage time exceeds three to four generations of the pest. Beans normally contain 1–2% of an  $\alpha$ -amylase inhibitor as well as various protease inhibitors. Similar levels of an amylase inhibitor, with or without E-64 (a tripeptide protease inhibitor from the mold, *Aspergillus japonicus*), were found to give substantial insect control in this study. Such levels of accumulation can be achieved when transgenes are expressed using strong seed-specific promoters that drive the expression of storage proteins in developing seeds.

Some proteinase inhibitor genes already have been expressed in plants, and the transgenic plants exhibited elevated resistance to insect attack (Hilder *et al.*, 1990; Hendriks *et al.*, 1991). However, Fernandes *et al.* (1993) reported that resistance of some cowpea lines was not correlated with the level of a proteinase inhibitor. Piergiorganni *et al.* (1991) demonstrated that certain bruchid-resistant cowpea lines had high levels of both proteinase inhibitors and  $\alpha$ -amylase inhibitors. Therefore, manipulation of combinations of genes that code for not only proteinase inhibitors but also  $\alpha$ -amylase inhibitors, such as those examined in this study, could be an effective insect control strategy. Use of two or more insect growth-inhibiting proteins that exert different mechanisms of action could lead to more durable host plant resistance to insects.

The seed inhibitors examined here had more potent effects on insect  $\alpha$ -amylases than human amylase. This result appears to confirm the hypothesis that these inhibitors evolved to slow the growth of insects feeding on the seeds. The purified inhibitors, when added to insect diets under laboratory conditions and in concentrations typical of their abundance in seeds, had adverse effects on the growth and development of insects. These results are significant because it is technically feasible to express these inhibitors in transgenic grain, where they could function to inhibit insect growth. We recently transformed rice with the gene of the bean amylase inhibitor driven by a rice endosperm promoter and will obtain transgenic seeds in the near future. Those seeds will be screened for resistance to stored grain insect pests.

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