Relative Toxicity of the Maize Endosperm Ribosome-Inactivating Protein to Insects

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The relative toxicity of proenzyme and protease-activated forms of maize seed ribosome-inactivating protein (b-32) to several insect species was determined. Only the protease-activated form had significant toxicity to any caterpillars when fed in diets at 1 mg/g of diet. Activity ranged from 70% mortality to cabbage looper (*Trichoplusia ni*) to no effect to Indian meal moth (*Plodia interpunctella*). Neither form of the protein showed activity against larvae of the Freeman sap beetle, (*Carpophilus freemani*). However, the proenzyme and protease-activated forms were approximately equally deterrent in choice assays to other sap beetles and maize weevils (*Sitophilus zeamais*), with relative feeding rates reduced by up to 6-fold. Because this protein can naturally occur at the 1 mg/g endosperm range in the endosperm of *Opaque-2* (normal) plants versus 2 orders of magnitude lower in *opaque-2* mutants, it is likely that this RIP plays a natural defensive role against insects. However, some insects appear to have adapted to this protein.

Keywords: *Host plant resistance; corn; Zea; Carpophilus; Helicoverpa; Spodoptera; Ostrinia; Sitophilus; Plodia; b-32*

INTRODUCTION

Maize (*Zea mays* L.), an agricultural grain crop of worldwide significance, is known to produce a wide variety of allelochemicals with activity against insects, including phenolics, flavonoids, and hydroxamates [e.g., Dowd and Vega (1996)]. More recently, several proteins that also appear to have activity against insects have been identified from maize. Some of these are tissue-specific, whereas others are distributed throughout different plant tissues. For example, proteins of the same type/class known to have activity against insects have been isolated from maize. They include proteases (Mitsuhashi and Oaks, 1994), protease and amylase inhibitors (Swartz et al., 1977; Mahoney et al., 1984; Blaco-Labra et al., 1995) tritin-like proteins (Coleman and Roberts, 1982), and chitinases (Neucere et al., 1991; Zhe-fu et al., 1992). However, studies that relate naturally occurring concentrations to defensive activity have been limited.

Several species of plants are known to produce ribosome-inactivating proteins (RIPs), which are specific *N*-glycosidases that can inactivate ribosomes by removing a specific adenine residue from the large rRNA (Stirpe and Barbieri, 1986; Endo et al., 1987; Stirpe et al., 1992; Barbieri et al., 1993). These RIPs have various degrees of toxicity both in vivo and in vitro. Ricin, an RIP from castor beans (*Ricinus communis*), is highly cytotoxic, but RIPs from edible grains such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) have no reported toxicity in vivo (Stirpe and Barbieri, 1986; Stirpe et al., 1992).

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Because of their ability to affect a fundamental process in cells, RIPs are thought to have a protective function in plants. Barley RIP synergized the inhibitory effect of chitinase and (1,3)-β-glucanase on the growth of *Trichoderma resei* and *Fusarium sporotrichoides* (Leah et al., 1991). The barley RIP expressed in tobacco (*Nicotiana tabacum*) protected plants from the soil-borne pathogen *Rhizoctonia solani* (Logemann et al., 1992; Jach et al., 1995). RIPs from seeds of dicots such as castor bean, soapwort (*Saponaria officinalis*), and bitter gourd (*Momordica cochinchinensis*) were toxic to beetles (Gatehouse et al., 1990) but not caterpillars (Gatehouse et al., 1990; Brandhorst et al., 1996).

In maize, an unusual RIP is synthesized specifically in kernel endosperms as a relatively inactive 32 kDa proenzyme (proRIP) (Walsh et al., 1991; Bass et al., 1992). NH_2-terminal, COOH-terminal, and internal acidic domains can be enzymatically removed from proRIP to yield two chains that interact noncovalently to form a much more active enzyme (Walsh et al., 1991; Bass et al., 1992). The activated form inhibits translation in a cell-free, rabbit reticulocyte system with an ID_{50} of 28−60 pM, at least 10000 times more active than the proRIP (Walsh et al., 1991).

The production of the maize proRIP is regulated by the *Opaque-2* locus (Soave et al., 1981; Bass et al., 1992). This protein occurs at relatively low levels in *opaque-2* mutant lines (Soave et al., 1981; Bass et al., 1992), which are also generally more susceptible to insect attack (Gupta et al., 1970). The association of increased insect susceptibility with RIP deficiency in *opaque-2* lines has led to suggestions that the RIP can play a defensive role against insects (Bass et al., 1992). The activation of maize proRIP by endogenous proteases occurs during germination, a time when storage protein reserves would be most vulnerable to pests and patho-

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gens (Hey et al., 1995). We now report on the relative toxicity of the proRIP and activated RIP to a series of insects and present further evidence for a natural defensive role of these proteins.

MATERIALS AND METHODS

Plants. Maize inbred W64A and its near isogenic mutants *opaque-2* and *floury-2* were grown at the Central Crops Research Unit in Clayton, NC. Ears were harvested at maturity or at 24 days after pollination. Immature ears were rapidly frozen in liquid nitrogen. Frozen kernels were shelled onto dry ice and stored at −80 °C.

Insects. Corn earworms (*Helicoverpa zea*) and fall armyworms (*Spodoptera frugiperda*) were reared on pinto bean based diet as described previously (Dowd, 1988). European corn borers (*Ostrinia nubilalis*) and cabbage loopers (*Trichoplusia ni*) were obtained from M. R. McGuire of NCAUR. Indian meal moths (*Plodia interpunctella*) were obtained from W. E. Burkholder (USDA−ARS, Madison WI, retired) and reared as larvae on Purina Layena chicken feed. Freeman sap beetles (*Carpophilus freemani*) and strawberry sap beetles (*Stelidota geminata*) were reared on pinto bean based diet as described previously (Dowd and Weber, 1991). Maize weevils (*Sitophilus zeamais*) were reared on cracked corn and brewer's yeast as described previously (Dowd, 1994). Dusky sap beetles (*Carpophilus lugubris*) were field collected and maintained on diet as described previously (Dowd, 1994). All insects were held at 27 ± 1 °C and 40 ± 10% relative humidity with a 14:10 h photoperiod except *S. zeamais*, which were reared at 60 ± 10% relative humidity.

Preparation of RIP. Kernels frozen at −80 °C were ground to a fine powder as described previously (Bass et al., 1992). ProRIP used in assays was purified from normal kernels of the W64A inbred harvested at 24 days after pollination. ProRIP was purified as described previously (Bass et al., 1992) except that fractions containing proRIP were pooled and concentrated with Centricon-10 concentrators (Amicon, Inc., Beverly MA). Protein concentration was determined with the BCA reagent and bovine serum albumin as a standard according to the supplier's instructions (Pierce Chemical, Rockford, IL). The final proRIP solution was stored in 20 mM phosphate buffer, pH 6.8, at −80 °C.

Purified proRIP was activated by incubating with immobilized papain (Pierce). The papain beads were washed and resuspended in 20 mM potassium phosphate buffer (pH 7.0), containing 10 mM EDTA, and 20 mM cysteine according to the supplier's instructions. The stored proRIP was adjusted to 5 mg/mL and 10 mM EDTA and mixed 1:1 (v/v) with the papain beads. The proRIP/bead solution was gently agitated at 37 °C for 3 h, after which time the papain beads were removed by centrifugation. The supernatant containing the activated RIP was then dialyzed against 20 mM phosphate buffer (pH 6.8). Both proRIP and activated RIP were prepared for insect assays by concentrating as described above, dialyzing out the buffer, and placing material in a Speed Vac until dry. ProRIP and papain-activated RIP were subjected to electrophoresis through 15% sodium dodecyl sulfate−polyacrylamide gels under denaturing conditions and stained with Coomassie Blue R-250 to confirm the integrity of the starting material and the extent of the proteolytic cleavage (Bass et al., 1992). Relative activities of proRIP and papain-activated RIP were determined using rabbit reticulocyte translation inhibition assays as described previously (Bass et al., 1992).

The natural concentration of proRIP was needed to set up bioassays with relevant levels. The concentration of proRIP in mature kernels was determined with a quantitative immunoblot assay. Briefly, proteins from ground kernels were fractionated through SDS−polyacrylamide gels alongside known amounts of purified proRIP standards. Proteins were electrophoretically transferred to nitrocellulose filters and probed with anti-proRIP antibody and secondary antibody radiolabeled with ^{35}S (Amersham Corp, Arlington Heights, IL). Radioactive emissions of the filters were measured with a Molecular Dynamics PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Insect Bioassays. The diet used in most assays was based on the pinto bean based diet described previously (Dowd, 1988) except that 240 g of frozen sweet corn was substituted for the water-saturated pinto beans. A sweet corn based diet was used to more closely approximate the natural food source of most of the insect species tested. Any maize RIP in the sweet corn would be inactivated during the preparation by heating above 60 °C. After being prepared and dispensed as 5 mL quantities into 35 mL cups, the diet material was allowed to set up and cool. The diets were then lyophilized. The RIPs were dissolved in water at 4 mg/g, and the RIP solution was added to the dried diet in a 3:1 ratio by weight (final concentration of RIP would be 1 mg/g of diet). The diet was allowed to absorb water for 2 days at 4 °C, and then the diets were sectioned for assays. Distribution of the RIPs in the diet appeared to be fairly uniform on the basis of the similar or lower standard errors for weights of surviving insects fed diets containing the RIPs versus control diets (see Results). For *S. zeamais*, each 5 mL block was sectioned into 16 pieces, and for caterpillars, the same quantity was sectioned into 20 pieces. For caterpillars, sections of diet were placed in wells of 24 well tissue culture plates, along with a single newly hatched caterpillar per well (total of 20 caterpillars per treatment) (Dowd, 1988). Assays were run for 7−10 days, after which time mortality was determined and survivors were weighed. For assays with *C. freemani* larvae, assays were set up in the following no-choice situation with second-instar (∼0.75 mg) larvae. A single block of diet ($^1/_{16}$ of the 5 mL) was placed in a tight-fitting Petri dish (Falcon No. 1006, Beckton Dickinson Co., Forest Park, NJ) along with 20 of the larvae per treatment. Assays were run for 4 days, at which point larvae had reached prepupal stage, so mortality and weights were determined. For *S. zeamais* and sap beetles, three-way choice assays involving water only, proRIP, and activated RIP were set up in the Petri dishes, with 10 adults per dish and at least 8 dishes per assay. Relative feeding was determined for *S. zeamais* by counting feeding punctures as described previously (Brandhorst et al., 1996) after 2, 4, and 7 days. Relative feeding by sap beetle adults was determined using a 1−4 rating scale (Dowd, 1990) after 2, 4, and 7 days. Assays were analyzed for statistical significance using Chi square analysis for mortality data, analysis of variance for weight data, and paired, two-tailed *t*-test analysis for choice assays.

The effect of proRIP and activated RIP on *S. zeamais* when incorporated into a more natural material was also determined. Lyophilized endosperm powder from 24 day after pollination W64A *floury-2* and W64A *opaque-2* kernels was rehydrated and equilibrated to 25% moisture using water or solutions containing the RIPs as described above. Powder was pressed into ∼0.25 g pellets using a pellet press with a $^1/_4$ in. (82 mm) diameter piston (Parr Instrument Co., Moline, IL). Pellets were pressed with a uniform pressure by replacing the press arm with a torque-sensing wrench (Utica Tools, Orangeburg, SC). The torque wrench was adjusted to 75 ft/lb (105 nm), and when pressure was applied to this level, the wrench would partly release, signaling to stop pressure. Preliminary tests with pellets made from normal and *floury-2* endosperm powder (which contains comparable levels of RIP to normal type kernels; A. D. Mehta and R. S. Boston, unpublished data) indicated the *floury-2* pellets were more similar in physical properties to those pressed from *opaque-2*, so the *floury-2* powder was used for comparison. Pellets were placed horizontally in Petri dishes with three paper clips cemented inside, so that pellets would not roll. Assays were set up and evaluated as just described (with 10 insects per dish and at least 8 dishes per assay), except a 1−10 rating scale was used to evaluate feeding. Relative feeding on pellets pressed from powder from mature seed of W64A *floury-2* and W64A *opaque-2* with 10 dish replicates was also determined. Pressed pellet assays were terminated after 4 days. Assays were analyzed for statistical significance using paired, two-tailed *t*-tests.

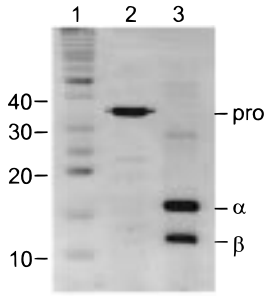

Figure 1. Polyacrylamide gel analysis of purified proRIP and papain-activated RIP. Left lane contains molecular weight markers, center lane contains purified proRIP, and right lane contains the α/β polypeptides of papain-activated RIP.

Table 1. Effect of Maize RIP on Caterpillars When Incorporated into Diet at 1 mg/g of Diet[a]

treatment	% mortality	survivor wt (mg)
H. zea		
control	0.0 a	17.4 ± 4.2 a
proRIP	10.5 a	15.6 ± 3.7 ab
activated RIP	0.0 a	7.9 ± 1.5 b
T. ni		
control	0.0 a	14.2 ± 2.2 a
proRIP	6.7 a	12.0 ± 3.3 a
activated RIP	71.4 b	1.7 ± 0.7
S. frugiperda		
control	11.8 ab	11.7 ± 1.8 ab
proRIP	5.3 a	14.3 ± 2.3 a
activated RIP	38.9 b	1.1 ± 0.3 b
O. nubilalis		
control	31.2 a	2.7 ± 0.6 a
proRIP	31.6 a	2.4 ± 0.5 ab
activated RIP	26.3 a	1.5 ± 0.2 b
P. interpunctella		
control	0.0 a	1.5 ± 0.1 a
proRIP	0.0 a	1.4 ± 0.1 a
activated RIP	0.0 a	1.4 ± 0.1 a

[a] Values are means ± standard errors. Values in columns of like insects followed by the same letter are not significantly different at $P < 0.05$ by Chi square (mortality) or analysis of variance (weights and punctures).

RESULTS

Treatment of proRIP with papain successfully produced an activated RIP (Figure 1) that was at least 100 times more active than the proRIP in translational inhibition assays in vitro (data not shown). ProRIP occurred at ~1.5 mg/g endosperm in normal W64A endosperm but at <0.010 mg/g endosperm in W64A *opaque-2* endosperm.

Only the papain-activated RIP was significantly active against caterpillars (Table 1). The caterpillar species varied in susceptibility to the active RIP and ranged (in order of increasing susceptibility compared to controls) *Plodia interpunctella* (weight $P = 0.537$) < *H. zea* (weight $P = 0.012$), *O. nubilalis* (weight $P = 0.036$) < *S. frugiperda* (weight $P = 0.000$) < *T. ni* (mortality $P = 0.000$).

In contrast to the effect on caterpillars, neither the proRIP nor the active RIP had any significant effect on either mortality or weight of survivors of *C. freemani* larvae (data not shown). However, both the proRIP and active RIP significantly deterred feeding in choice assays against adult *C. freemani*, *C. lugubris*, *S. geminata*, and *S. zeamais* (Table 2). In several cases, the proRIP inhibited feeding by as much as the activated RIP did.

The *S. zeamais* feeding on mature *floury-2* pellets was significantly less than on mature *opaque-2* pellets (Table 3). This trend was reversed to some extent using pellets made from endosperm harvested 24 days after pollination (Table 3). However, the *opaque-2* pellets containing both the proRIP and active RIP were fed on only to a limited extent compared to the other types of pellets. The ratio of feeding on the *opaque-2* pellets with and without the RIPs was similar to that seen with sweet corn based artificial diets.

Table 2. Effect of Maize RIP on Beetles When Incorporated into Diet at 1 mg/g of Diet in Choice Assays

	feeding activity[a]		
treatment	day 2	day 4	day 7
S. zeamais			
control	3.3 ± 0.4 a	12.2 ± 1.5 a	37.1 ± 2.1 a
proRIP	2.1 ± 0.8 b	3.4 ± 0.5 b	7.9 ± 1.0 b
activated RIP	0.8 ± 0.2 c	1.1 ± 0.2 c	7.1 ± 1.1 b
C. freemani			
control	ND	1.9 ± 0.2 a	2.9 ± 0.2 a
proRIP	ND	0.7 ± 0.2 b	2.2 ± 0.2 b
activated RIP	ND	0.4 ± 0.2 b	2.1 ± 0.2 b
C. lugubris			
control	1.5 ± 0.1 a	2.5 ± 0.2 a	3.1 ± 0.2 a
proRIP	0.1 ± 0.1 b	0.2 ± 0.1 b	0.5 ± 0.2 b
activated RIP	0.1 ± 0.1 b	0.7 ± 0.2 c	1.1 ± 0.2 c
S. geminata			
control	1.6 ± 0.2 a	2.4 ± 0.2 a	2.5 ± 0.2 a
proRIP	0.8 ± 0.1 b	1.1 ± 0.2 b	1.2 ± 0.2 b
activated RIP	0.8 ± 0.1 b	1.4 ± 0.2 b	1.8 ± 0.1 c

[a] Values are means ± standard errors for 16 replicates. Values in columns followed by the same letter are not significantly different at $P < 0.05$ by paired, two-tailed *t*-tests. ND, not determined due to limited feeding on all treatments. Results for *S. zeamais* are given on the basis of number of punctures; those for the remaining treatments are rated on a 1−4 scale.

Table 3. Effect of Maize RIP Incorporated into Endosperm Powder Pellets on *S. zeamais* When Incorporated at 1 mg/g of Pellet in Choice Assays

	feeding activity[a] (1−10 scale)	
treatment	day 2	day 4
pellets of powder from mature kernels		
floury-2	1.3 ± 0.3 a	1.7 ± 0.4 a
opaque-2	2.8 ± 0.2 b	3.6 ± 0.3 b
pellets of powder from kernels 24 days after pollination		
proRIP		
floury-2	2.6 ± 0.4 a	4.2 ± 1.6 a
opaque-2	1.6 ± 0.2 b	2.8 ± 0.2 b
opaque-2 + RIP	0.6 ± 0.2 c	0.9 ± 0.1 c
papain-activated RIP		
floury-2	2.5 ± 0.3 a	3.9 ± 0.5 a
opaque-2	1.6 ± 0.2 b	2.5 ± 0.3 b
opaque-2 + RIP	0.4 ± 0.2 c	0.8 ± 0.2 c

[a] Values are means ± standard errors for 8 (proRIP) or 10 (enzyme-activated RIP) replicates. Values in columns followed by the same letter are not significantly different at $P < 0.05$ by paired, two-tailed *t*-tests.

DISCUSSION

The presence of high (>1 mg/g endosperm) levels of RIP in mature *Opaque-2* compared to mutant *opaque-2* maize kernels appears to explain at least partly the resistance of normal versus mutant *opaque-2* lines of maize to stored product beetles, provided the corresponding lines are not confounded by various levels of other insect active proteins, such as the enzymes and enzyme inhibitors discussed previously. Because both mutants have pleiotropic phenotypes, other factors also may be responsible for deterring insect feeding in the *opaque-2* versus *floury-2* 24 DAP endosperms.

Plant RIPs are generally relatively inactive toward insect cell cultures and whole insects (Barbieri et al., 1993). However, high activity of ricin and saporin was reported against the beetles *Anthonomus grandis* and *Callosobruchus maculatus* but not against the caterpillars *Spodoptera littoralis* and *Heliothis virescens* (Gatehouse et al., 1990). Our results indicate the protease-activated form of the maize RIP, which is the form molecularly homologous to most other plant RIPs, was active against both beetles and caterpillars at the same concentrations.

Midguts of beetles are typically mildly acidic, whereas those of caterpillars are moderately alkaline (Purcell et al., 1992). Although degradation/activation of the maize RIPs could be occurring under the alkaline conditions in the caterpillar midguts, no breakdown/activation of the maize RIPs has been noted at similar pH values over a 24 h period (S. Jeyarajah and R. S. Boston, unpublished data). However, the maize RIP could be readily activated by a cysteine protease (in this case papain). Cysteine proteases are not reported in caterpillars such as *H. zea* (Purcell et al., 1992). Cysteine proteases are present in several beetle species (Purcell et al., 1992; Wolfson and Murdock, 1990) including *S. zeamais* (Houseman and Thie, 1993) and the sap beetle *Glischrochilus quadrisignatus* (Murdock et al., 1987). As previously discussed, other plant RIPs were acutely toxic to beetles (Gatehouse et al., 1990). However, maize RIP also appears to have an antifeedant effect, which has been reported previously only for the fungal RIP restrictocin (Brandhorst et al., 1996). The presence of cysteine proteases in *S. zeamais*, together with results showing activation of proRIP by the cysteine protease papain, suggests the *S. zeamais* gut enzymes may activate the proRIP. Incubation of *S. zeamais* gut homogenates with proRIP resulted in the production of a cationic protein from the anionic proRIP (the enzyme-activated maize RIP is also cationic), but relative activity in vitro with translational inhibition assays have not yet been performed (Dowd et al., unpublished data). Low activity of other plant RIPs to caterpillars relative to beetles appears to be related to greater rates of total digestion by caterpillars (Gatehouse et al., 1990). The papain-activated form of maize RIP appears to be as resistant to total digestion by adversely affected caterpillar species tested (*T. ni*, *H. zea*, *S. frugiperda*) as it is to total digestion by *S. zeamais* (Dowd et al., unpublished data).

Caterpillar susceptibility to the activated maize RIP appeared to be related to host adaptation (or lack thereof). The *T. ni*, which do not feed on maize, were most severely affected. Although often a pest of maize ears, *S. frugiperda* is more common as a leaf feeder, while *H. zea* and *O. nubilalis* are commonly seen in ears feeding on maize kernels (Metcalf and Metcalf, 1993). The *P. interpunctella* commonly feed on stored maize (Metcalf and Metcalf, 1993) and, so, would be expected to have adapted to the endosperm protein present in the mature maize. The resistance of *O. nubilalis* to maize-derived versus other protease inhibitors has also been attributed to host adaptation (Larocque and Houseman, 1990).

Expression of the maize endosperm-specific RIP is controlled by the transcriptional activator *Opaque-2* (Bass et al., 1992). We have found that the proRIP concentration in the mutant W64A inbred *opaque-2* is ~100-fold less than in the normal type kernels. Several reports have indicated *opaque-2* maize is more susceptible to attack by insects and ear-rotting fungi than corresponding normal lines (Gupta, 1970; Warren, 1978; Gulya et al., 1979). The pleiotropic phenotype of *opaque-2* has made it difficult to separate susceptibility to insects from other properties such as soft endosperm and other potential antiinsectan allelochemicals or proteins.

Although active against nonsource ribosomes, relevant levels of proRIP or activated maize RIP do not affect maize ribosomes (Bass et al., 1992; Hey et al., 1995). Thus, genetic engineering of expression of the enzyme-activated form in maize is a plausible means for increasing resistance to both beetle and caterpillar pests. Our work, with purified RIP used at naturally occurring concentrations, demonstrates that it can play a natural defensive role against some relevant insect species, but insects species continually exposed to it based on feeding preference have apparently adapted to it (including the protease-activated form). The maize RIP is an example of a proteinaceous defensive compound associated with resistance to both plant pathogens (Cleveland et al., 1994) and insects, a multifunctionality that also has been described for some maize allelochemicals (Dowd, 1990).

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