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Potentiation of *Bacillus thuringiensis* Insecticidal Activity by Serine Protease Inhibitors

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Several serine protease inhibitors enhanced the insecticidal activity of the insect control proteins from *Bacillus thuringiensis* var. kurstaki, var. tenebrionis, and var. israelensis against their target insects, tobacco budworm and other lepidopterans, Colorado potato beetle, and mosquito, respectively. *B. thuringiensis* protein concentrations, at levels causing minimal insect mortality (10–20%), were mixed with purified protease inhibitors and assayed against the appropriate target insects. The presence of extremely low levels (4 μM) of protease inhibitors enhanced the insecticidal activity of *B. thuringiensis* var. kurstaki by 2–20-fold. Protease inhibitors also potentiated the insect growth inhibitory activity of sublethal *B. thuringiensis* var. kurstaki protein concentrations. Genetically improved tobacco plants expressing a protease inhibitor fused to a truncated *B. thuringiensis* insect control protein showed levels of activity enhancement similar to those seen with purified protease inhibitors.

A variety of *Bacillus thuringiensis* (Bt) strains have been isolated, which produce proteins active against a wide range of insects including lepidopterans, coleopterans, and dipterans (Klausner, 1984). Bt is a Gram-positive, spore-forming bacterium that characteristically produces a parasporal crystal protein that accounts for this insecticidal activity. Crystal and spore preparations of the lepidopteran-active *B. thuringiensis* var. kurstaki (Btk) have been used as commercial insecticides for many years. A second class of Bt proteins, produced by strains exemplified by *B. thuringiensis* var. israelensis (Bti), are active against dipteran insects (Thomas and Eller, 1983; Tyrell et al., 1979). Recently, a new class of Bt proteins, active against coleopteran insects, was isolated from *B. thuringiensis* var. tenebrionis (Btt) (Krieg et al., 1983) and *B. thuringiensis* var. san diego (Btsd) (Herrnstadt et al., 1986). Classification of these three crystal protein types Btk, Btt, and Bti are identified as cryI, cryIII, and cryIV, respectively (Höfte and Whiteley, 1989).

All three classes of Bt proteins have extremely short

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half-lives when applied topically (Beegle et al., 1981), due in part to their sensitivity to inactivation by ultraviolet light (Pozsgay et al., 1987). An improved method to deliver the Btk, Btt, or Btsd proteins was developed by genetically improving the genes, which encode these proteins, directly into plants. Plants have been transformed and regenerated that express either the Btk (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987) or Btt (Fischhoff et al., unpublished work) proteins at insecticidal levels.

Although genetically improved plants that express Btk proteins are insecticidal to sensitive insects, quantitative data (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987) show that these proteins are expressed at low levels. These levels may not provide effective control for some agronomically important pests (e.g., *Heliothis zea*, corn earworm, or *Spodoptera exigua*, beet armyworm), which are relatively insensitive to the Btk protein. Various methods are currently being developed to enhance the expression or unit activity of Bt proteins in plants. Increasing the in planta activity of Bt protein by coexpression of other proteins has a number of advantages. In addition to enhancing the insecticidal efficacy, proteins with multiple modes of action may extend the insect host range spectrum or circumvent the development of insect resistance.

Another class of proteins shown to have insecticidal activity are plant protease inhibitors. Serine protease inhibitors are the most abundant and well-characterized class of protease inhibitors. Levels of plant protease inhibitors are high in legume seeds, 1–4% of the total protein. These inhibitors contain at least one or more peptide bond(s) known as the reactive site, which interact with the active site of serine proteases to form an enzyme–substrate complex. Two types of soybean protease inhibitors, Kunitz and Bowman–Birk, were studied in detail in this report. The Kunitz inhibitor is a single-headed trypsin inhibitor with a molecular weight of 24 000. The Bowman–Birk inhibitor has dual inhibitory activity against both trypsin and chymotrypsin with a molecular weight of 8000. The role of these protease inhibitors in plant seeds has been speculated for many years. Protease inhibitors may have evolved as plant defense mechanisms against herbivores (Ryan, 1981). Numerous groups have studied the effects of feeding plant protease inhibitors to insect larvae and found that some of these inhibitors can be strong deterrents to insect attacks and are, in fact, insecticidal themselves at high concentrations (Birk and Applebaum, 1960; Gatehouse and Boulter, 1983; Hilder et al., 1987). It is important to note that an insecticidal effect is observed only at very high inhibitor concentrations, 2–5% of the total diet (Gatehouse and Boulter, 1983; Hilder et al., 1987). Growth inhibitory effects of protease inhibitors at lower concentrations, 0.18%, on corn earworm (*H. zea*) and beet armyworm (*S. exigua*) are reported by Broadway et al. (1986).

In this report insecticidal efficacy of various Bt proteins and protease inhibitors alone and in combination were bioassayed against their respective target insects. The Bt protein concentrations were adjusted to levels appropriate to study either mortality or growth inhibition. We demonstrate that serine protease inhibitors enhance the activity of all three classes of Bt proteins, Btk, Btt, and Bti, against tobacco budworm and other lepidopterans, Colorado potato beetle, and mosquito, respectively. The enhanced activity was defined as potentiation since none of the protease inhibitors had insecticidal activity when tested alone. This potentiation occurred at greater than 10^5 times lower serine protease inhibitor concentrations than those reported to be insecticidal. The potentiating effect of serine protease inhibitors on Bt activity extends from in vitro responses utilizing purified proteins to an in planta demonstration with genetically improved tobacco plants containing a protease inhibitor–Bt fusion protein.

METHODS AND MATERIALS

Reagents. Trypsin, excision grade, was obtained from Calbiochem, La Jolla, CA. Sepharose 4B-CL, cyanogen bromide, benzoyl-DL-arginine *p*-nitroanilide (BAPNA), and all other reagent grade chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Anhydrotrypsin–Sepharose was prepared as described (Ishii et al., 1983). The commercial Btk HD-1 insecticide Dipel and the Bti insecticide Vectobac were obtained from Abbott Laboratories, North Chicago, IL.

Protease Inhibitors. Two classes of soybean inhibitors, Kunitz (trypsin inhibitor) and Bowman–Birk (trypsin–chymotrypsin inhibitor), were obtained from Sigma. Both the Kunitz and Bowman–Birk inhibitors were further purified and the N-terminal amino acid sequences determined (Hewick et al., 1981) to substantiate purity and confirm their identity by comparison with published amino acid sequences.

The Kunitz inhibitor was further purified by elution as a single major peak from a Superose-12 FPLC column (HR10/30; Pharmacia AB, Uppsala, Sweden) in 100 mM sodium phosphate–200 mM sodium chloride (pH 6.8). A purity of >95% was estimated by integration of the Superose-eluted peak and confirmed by N-terminal amino acid sequencing. The sequence determined from the first 15 amino acids agreed completely with the sequence reported for the soybean Kunitz trypsin inhibitor (Koide and Ikenaka, 1972).

The Bowman–Birk inhibitor was further purified on a FPLC Mono Q (HR5/5) column (Pharmacia AB). The sample was dissolved in 20 mM piperazine (pH 5.0) and applied to and eluted with a linear gradient of 0–300 mM sodium chloride in the same buffer (Hwang et al., 1977). A single major peak confirmed that the purity was >95%. The N-terminal 15 amino acids sequence agreed completely with the published sequence of the soybean Bowman–Birk inhibitor (Odani and Ikenaka, 1978).

Additional trypsin inhibitors from bovine pancreas and ovomucoid (chicken egg white) were obtained from Sigma and used without further purification. A 29 amino acid squash (*Curcurbita maxima*) trypsin protease inhibitor (CMTI) (McWherter et al., 1989) was chemically synthesized and provided by C. McWherter (Monsanto Co.).

Chromogenic Activity. Trypsin inhibitory activity of the various protease inhibitors was determined with use of a chromogenic peptide *p*-nitroanilide substrate (BAPNA) (Fritz et al., 1974). Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

Preparation of Seed Extracts and Protease Inhibitors. Seeds from the plants specified in Figure 1 were ground to a coarse powder, and the inhibitors were extracted at 4 °C in 100 mM sodium phosphate–150 mM sodium chloride (pH 7.0) buffer (1/5, w/v). After being mixed overnight, the particulates were removed by filtering the extracts through several layers of cheesecloth and then centrifugation (14000*g*) for 20 min. Typically, 20 mL of the crude seed extracts was prepared for insect bioassay and related assays. Larger batches (100 mL) were prepared for purification purposes. Protein concentrations ranged from 5 to 15 mg/mL or 25 to 75 mg/g of seed. Trypsin inhibitory activity ranged from 0.1 to 0.5 mg of trypsin inhibited/mg of total protein.

Serine protease inhibitors were purified from crude redbean and cowpea seed extracts by concentrating the protein by ammonium sulfate precipitation (80%). The precipitate was dissolved in the original volume of deionized water and dialyzed in a spectrapor membrane (Amicon, Danvers, MA; molecular weight cutoff 6000–8000) against 50 mM sodium acetate–20 mM calcium chloride (pH 5.1). The reduced pH caused a visible precipitate that was removed by centrifugation and discarded. The supernatant was subjected to affinity chromatography on

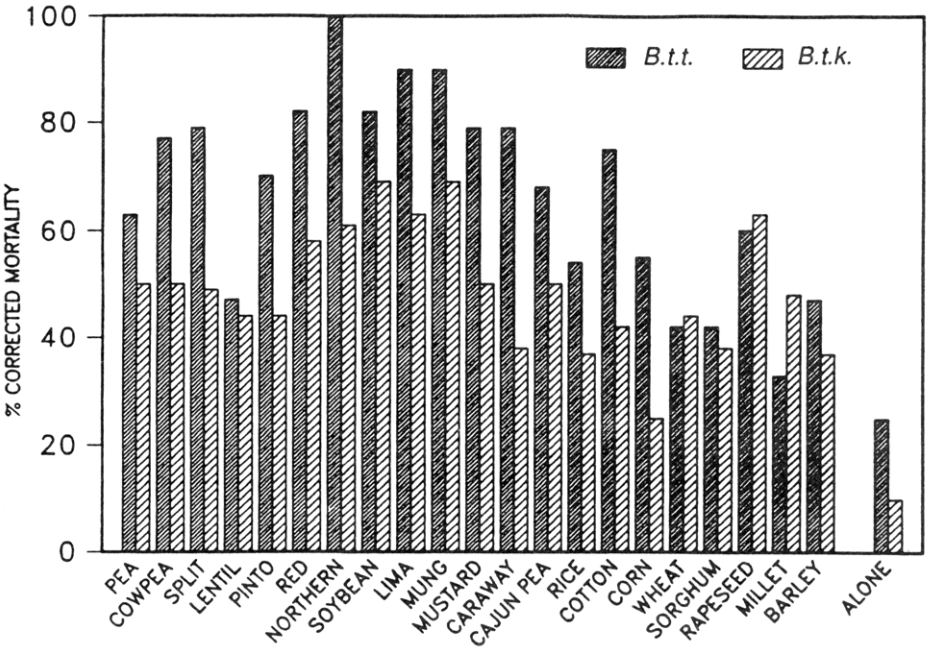

Figure 1. Potentiation of Bt proteins by crude seed extracts. Seed extracts were prepared and used undiluted as described in Methods and Materials. Concentrations of Btt and Btk HD-73 proteins were 2.5 and 0.5 mg/mL, respectively, which caused only 10–20% insect mortality.

an anhydrotrypsin–Sepharose column as described by Ishii (1983). Eluted fractions with the highest absorbance at 280 nm were pooled and assayed for inhibitory activity.

Purification of Bt Insecticidal Proteins. Btt protein (cryIIIA) was purified from *Escherichia coli* JM101 (pMON5456) (McPherson et al., 1988) (S. MacIntosh, unpublished results).

Btk HD-1 protein (cryIA(b)) was isolated from an *E. coli* strain containing a plasmid that encodes the full-length Btk protein comparable to pMAP4 (Fischhoff et al., 1987). This HD-1 gene was isolated from the Btk HD-1 strain that contains at least three different Btk genes (Wilcox et al., 1986). Cultures were grown, induced, and harvested and protein inclusions isolated. After sonication and centrifugation, the inclusion bodies were extensively washed with water and the Btk protein solubilized in 100 mM sodium carbonate–10 mM dithiothreitol (DTT) buffer (pH 10). The clear supernatant was precipitated with 40% ammonium sulfate, dissolved in 100 mM sodium carbonate–10 mM DTT buffer (pH 10), and subjected to ion-exchange chromatography on a Mono Q (HR10/10) column (Pharmacia) utilizing a 0–1 M potassium thiocyanate gradient.

Protein crystals containing the full-length Btk HD-73 protein (cryIA(c)) were purified by isopycnic centrifugation in sodium diatrizoate gradient as described (Ang and Nickerson, 1978; Nickerson, 1981) and provided by P. Lavrik (Monsanto). The lyophilized crystals were solubilized in 100 mM sodium carbonate–10 mM DTT (pH 10).

Btk HD-73, *Trichoplusia ni* cleaved protein was prepared from full-length Btk HD-73 by the procedure of Yamamoto and Odoni (1983) and provided by C. C. Beegle (Cotton Insects Research, USDA, Brownsville, TX).

Both HD-73 and HD-1 Btk proteins were tested for insecticidal activity as full-length and truncated (trypsinized) forms. Trypsinolysis was performed on the full-length Btk HD-1 and HD-73 proteins described above. Following dialysis into 100 mM sodium carbonate (pH 10) to eliminate high salts and reducing agents, trypsin was mixed with Btk protein in a 1/5 (w/w) ratio and incubated at 18 °C for 6 h. The solution was stored at 4 °C and the extent of cleavage (conversion to the tryptic resistant fragment) determined by SDS-PAGE analysis (Laemmli, 1970). Once the reaction was determined to be complete, the truncated Bt protein was extensively dialyzed against 100 mM sodium carbonate (pH 10).

In Planta Expression of a Fused Protein, CMTI–Btk HD-1. A synthetic DNA fragment was designed on the basis of the amino acid sequence of the mature CMTI protein sequence (McWherter et al., 1989). The DNA fragment was cloned as a BglII–ScaI fragment upstream of the Btk HD-1 gene after site-directed mutagenesis of Btk HD-1 gene to insert a BglII site and a ScaI site in frame with CMTI at amino acid 26 of Btk HD-1. A BglII site was also inserted at the C-terminal portion of the coding sequence just downstream of the translational termination signal so that the DNA coding sequence could be removed as a BglII fragment. The resulting CMTI–Btk HD-1 hybrid coding sequence contains 29 amino acids of CMTI fused to amino acids 26–612 of Btk HD-1. This DNA fragment was subcloned into pMON7259, a pUC-like vector with a BglII site within the multilinker in the same orientation as the β-galactosidase gene (pMON5381). The CMTI–Btk HD-1 fused protein expressed in *E. coli* containing pMON5381 was tested for toxicity in insect bioassays. The BglII fragment of pMON5381 was cloned into a plant expression vector for *Agrobacterium*-mediated transformation and regeneration of transgenic tobacco plants, performed as described (Fischhoff et al., 1987).

The insecticidal efficacy of tobacco plants expressing either the Btk HD-1 protein or the CMTI–Btk HD-1 fusion protein were compared in a diet incorporation bioassay with plant extracts prepared by grinding frozen leaf tissue in 100 mM sodium carbonate (pH 10) in a ratio of 1 g of tissue to 2 mL of buffer. Five serial dilutions of the extracts were incorporated into the artificial diet of the tobacco budworm. The final concentration of plant total protein within the diet was 0.7 mg/mL for the highest concentration tested of each plant extract. The Btk protein concentration of each plant extract was determined by western analysis (Towbin et al., 1979). The fold increase in activity was calculated by averaging the data from each pair of plants with and without CMTI protein and then comparing the actual Btk protein level to the effective level caused by the presence of the CMTI fusion protein.

Insect Bioassays. Btt activity was assayed with newly hatched Colorado potato beetle, *Leptinotarsa decemlineata*, larvae in tomato leaf feeding assay (McPherson et al., 1988). Samples of Btt alone, inhibitor alone, or combinations of the two proteins were prepared on the day of the test. Btk activity (HD-1 and HD-73) was typically assayed with neonate *Heliothis virescens*, tobacco budworm, larvae in an artificial diet assay, comparable to that described by Marrone et al. (1985). The same diet incorporation assay was also used to examine the effect of the protease inhibitors in combination with Btk protein on *H. zea*, corn earworm, *T. ni*, cabbage looper, and *Manduca sexta*, tobacco hornworm. The diet medium was prepared the day of the test with Btk protein solutions, crude seed extracts, or plant tissue extracts replacing the 20% water component of

the diet. One neonate larva was added to 1 mL of treated gelled diet in each well of an 8 × 11 in. insect diet tray. The trays were incubated at 27 °C, and the mortality was determined at 6 days. The percent corrected mortality was determined by the method of Abbott (1925). Data are presented as fold increase in activity as it relates to Bt protein concentration. A known dose of Bt protein in combination with protease inhibitor will cause a level of mortality (or larval growth reduction, see below) equivalent to a higher dose of Bt protein alone, as determined from a standard curve of Bt protein. This calculated dose of Bt protein is divided by the actual dose of Bt protein to give a fold increase in activity. A standard curve of Bt protein without the addition of protease inhibitor was included in every experiment. For experiments where the efficacy of Btk protein was measured in terms of larval growth reduction, larvae for each treatment were combined at day 7 and weighed on an analytical balance. The average weight per insect was calculated and compared to a standard curve relating Btk protein concentration to average larval weight. All insect bioassay data represent the average of at least three replicate experiments. Bti activity (Vectobac) was assayed with third-instar *Aedes aegypti*, yellow fever mosquito, larvae as described by McLaughlin et al. (1981). Mortality was determined at 7 days.

RESULTS

Crude plant seed extracts from more than 20 plants potentiated the insecticidal activity of Bt protein when fed in combination with either Btk or Btt protein at a dose causing minimal mortality (10–20%) to their respective target insects (Btk against tobacco budworm and Btt against Colorado potato beetle) (Figure 1). No decrease in potentiation of Bt activity was observed after the crude extracts were boiled for 5 min. Protease inhibitors are known to be heat stable due to the high degree of crosslinking by disulfide bonds (Ryan, 1981). The undiluted crude seed extracts alone showed no insecticidal activity, measured by mortality or growth reduction (data not shown). In particular, legume seeds containing relatively high levels of protein inhibitors (1–4% of total protein) (Ryan, 1983) showed the highest level of potentiation.

Several factors, including the protease inhibitors, within the seed have been shown to be insecticidal (Gatehouse et al., 1986; Shukle and Murdock, 1983). Therefore, purified soybean Kunitz and Bowman–Birk protease inhibitors were tested in combination with Btt protein against Colorado potato beetle to determine whether they were, at least in part, responsible for the potentiation. A concentration of Btt protein (2.5 μg/mL) was used that caused minimal mortality (about 20%). The Kunitz inhibitor potentiated Btt insecticidal activity 8–40-fold (Figure 2). For instance, the combination of 10 mg/mL Kunitz inhibitor with 2.5 μg/mL Btt protein produced mortality equivalent to approximately 100 μg/mL Btt protein or a 40-fold (2.5 to 100 μg/mL) potentiation of Btt activity. The Bowman–Birk inhibitor had a significant but less dramatic effect (data not shown). Both soybean inhibitors when tested alone were not insecticidal even at the highest level tested (10 mg/mL or 6.4% of diet).

These soybean protease inhibitors also increased the insecticidal activity of the lepidopteran-active toxin, Btk HD-73, protein (*T. ni* cleaved) against the tobacco budworm by 2–8-fold in an artificial diet assay. This insecticidal effect was observed as either an increase in mortality (Figure 3) or a decrease in insect larval weight (Table I). Insect weight was inversely proportional (in a logarithmic manner) to the relative increase in Btk protein concentration. A linear relationship was observed from 35% to approximately 90% growth reduction of tobacco budworm larvae as compared to a buffer standard. Btk protein levels were used that caused 10% insect mortality (about 0.5 μg/mL) or 40% larval growth reduction (0.004–0.05 μg/mL). Similar increases in Btk efficacy were observed with both mortality and larval growth reduction assays. The potentiating effect was observed at very low inhibitor concentrations (0.000 002–0.6%). Kunitz and Bowman–Birk inhibitors were equally effective in enhancing Btk insecticidal activity in combination with *T. ni* cleaved HD-73 Btk protein (Figure 3; Table I).

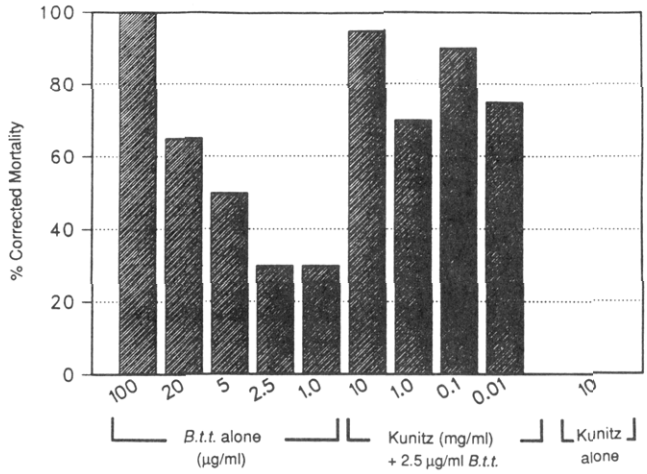

Figure 2. Insecticidal activity of Btt protein alone, soybean Kunitz inhibitor alone, and combinations of Btt protein and Kunitz inhibitor on the Colorado potato beetle, in a detached leaf bioassay.

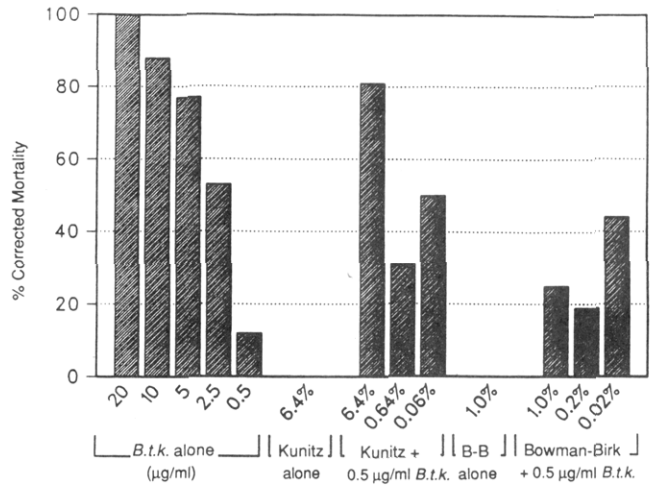

Figure 3. Insecticidal activity of Btk HD-73 protein (*T. ni* cleaved), soybean protease inhibitors, and combinations of Btk HD-73 protein and protease inhibitors on tobacco budworm larvae, in a diet incorporation bioassay.

Table I. Potentiation of Btk HD-73[a] Activity

inhibitor concn		
μM	%	inc in act., *x*-fold
	Bowman–Birk	
0.4	0.002	8.2
0.04	0.0002	6.9
0.004	0.00002	3.8
0.0004	0.000002	2.8
	Kunitz	
0.4	0.006	6.2
0.04	0.0006	6.2
0.004	0.00006	4.2
0.0004	0.000006	3.5

[a] Btk HD-73 protein concentration was 0.004 μg/mL (2.5×10^{-6}% of the diet). Activity was measured in a larval weight reduction assay of *H. virescens* (tobacco budworm).

Table II. Potentiation of Btk HD-73[a] Activity by Various Inhibitors

inhibitor[b]	inc in act., x-fold
soybean Kunitz[c]	3.6
soybean Bowman-Birk[c]	2.8
redbean	2.4
cowpea	1.8
squash	3.3
pancreatic	3.5
ovomucoid	2.0

[a] The Btk HD-73 (*T. ni* cleaved) protein was 0.088 μg/mL or 0.000 005% of the diet. Activity was measured in the weight reduction assay with *H. virescens* (tobacco budworm). [b] All inhibitors were tested at equivalent trypsin inhibitory activity as determined in a chromogenic assay with the soybean Kunitz inhibitor as the standard. [c] The concentrations of purified Bowman-Birk and Kunitz inhibitors were 0.037 mg/mL (0.02%) and 0.1 mg/mL (0.06% of the diet), respectively.

Table III. Potentiation of Btk Activity by Soybean Protease Inhibitors

type of Btk protein	Btk, μg/mL	inc in act.,[a] x-fold Bowman-Birk[b]	Kunitz[b]
HD-73 (full length)	1.0	1.3	2.1
HD-73 (*T. ni* cleaved)	0.01	2.8	3.6
HD-73 (tryptic)	0.1	1.7	1.7
HD-1 (full length)	1.0	1.3	3.4
HD-1 (tryptic fragment)	0.01	3.0	3.4
DIPEL	20	2.3	3.2

[a] Activity was measured in a larval weight reduction assay with *H. virescens* (tobacco budworm). A Btk protein concentration was chosen that caused only minimal insect weight loss. [b] The soybean Bowman-Birk and Kunitz inhibitors were tested at concentrations of 0.037 mg/mL (0.02%) and 0.1 mg/mL (0.06% of the diet), respectively.

Purified cowpea and redbean seed inhibitors, synthetic CMTI protein, and the animal protease inhibitors from pancreatic and ovomucoid sources also enhanced the activity of Btk protein against tobacco budworm. The level of potentiation, as measured by larval growth reduction, was similar to that observed with the soybean inhibitors when the inhibitors were tested at equivalent in vitro trypsin inhibitory activity (Table II). No larval growth reduction was observed with any of these inhibitors alone. Although some of the inhibitors in Table II are known to have inhibitory activity against chymotrypsin (Bowman-Birk; Ryan, 1981), only inhibitors with antitrypsin activity potentiated Bt activity. A wide range of inhibitors representing classes including metallo, sulfhydryl, carboxyl, and amylase had no potentiating effects when tested in combination with Bt proteins.

Additional Btk proteins were tested with the soybean protease inhibitors to examine the range of Bt proteins potentiated by the tobacco budworm larval growth reduction assay. Dipel was tested in addition to the purified full-length forms of Btk HD-1 and HD-73 proteins and their respective tryptic fragments. With limited exceptions, the inhibitors potentiated the activity of the Btk proteins by 2–4-fold (Table III). The activity of the full-length HD-1 and HD-73 proteins was only slightly increased by the soybean Bowman-Birk inhibitor but was significantly potentiated by the soybean Kunitz inhibitor. Neither inhibitor potentiated the activity of the trypsinized form of the Btk HD-73 protein.

Vectobac, a Bt formulation containing a third type of Bt protein (Bti), was tested in combination with soybean protease inhibitors against the mosquito, *Aedes aegypti*. The Kunitz inhibitor potentiated Bti activity, estimated by mortality, 3-fold (data not shown). The Bowman-Birk inhibitor had no effect.

Table IV. Potentiation of Btk HD-73 Activity against a Variety of Lepidopterans

lepidopteran insect	Btk,[a] μg/mL	inc in act., x-fold Bowman-Birk[b]	Kunitz[b]
M. sexta (tobacco hornworm)	0.03	4.1	2.5
H. virescens (tobacco budworm)	0.01	2.8	3.6
T. ni (cabbage looper)	0.03	3.8	3.7
H. zea (corn earworm)	0.01	8.1	13.0

[a] The Btk HD-73 (*T. ni* cleaved) protein concentration indicated was the amount of Btk protein/mL of diet. Activity was measured in the larval weight reduction assay. [b] The soybean Bowman-Birk and Kunitz inhibitors were tested at concentrations of 0.037 mg/mL (0.02%) and 0.1 mg/mL (0.06% of the diet), respectively.

Three other lepidopteran insects, tobacco hornworm, corn earworm, and cabbage looper, were tested with combinations of the soybean protease inhibitors and Btk HD-73 (*T. ni* cleaved) protein to investigate the range of insects susceptible to Bt potentiation. Btk activity was potentiated in all insects, 2.5–13-fold (Table IV). The cabbage looper was the only insect examined that showed any direct insecticidal effects attributed to the soybean inhibitors. Minimal mortality (about 25%) and a 50% reduction in larval weight was observed with a 0.4% concentration of either Bowman-Birk or Kunitz inhibitor, in the absence of Btk proteins.

Extensive evidence was obtained that established protease inhibitor potentiation of the insecticidal efficacy of purified Bt proteins. However, the ultimate objective was to demonstrate in planta potentiation with the CMTI-Btk HD-1 fusion protein. A unique gene was designed by placing the inhibitor sequence upstream of amino acid 25 of the Btk gene. This maintained the Btk sequence, which is cleaved by insect gut proteases, between amino acids 28 and 29. The resulting fusion protein would then be cleaved by insect gut proteases and release both the insecticidally active Btk protein and the potentiating CMTI protein. Attempts to measure protease inhibitory activity in leaf extracts with the BAPNA substrate were unsuccessful because chlorophyll interfered with the chromogenic assay. Both sets of transformed tobacco plants, containing the Btk HD-1 protein or the fusion CMTI-Btk HD-1 protein, were of the same cultivar; the only difference is the presence of the protease inhibitor, CMTI. The total protein recovery of all the extracts was very consistent at 7.4 ± 0.6 mg of protein/g of plant tissue. Protein extracts prepared from two independent plants that express the fusion protein, CMTI-Btk HD-1, showed approximately a 6-fold increase in specific activity in the tobacco budworm larval growth reduction assay (see Methods and Materials) compared to extracts of the two plants that express only the Btk HD-1 protein (Figure 4).

DISCUSSION

Genes encoding Btk proteins have been expressed at low levels in transgenic plants. These transgenic plants exhibit insecticidal activity against sensitive insects (e.g., tobacco hornworm) (Barton et al., 1987; Fischhoff et al., 1987; Vaeck et al., 1987), but the efficacy against some agronomically important, less sensitive, pests (e.g., corn earworm and beet armyworm) is less dramatic (Fischhoff et al., unpublished results). We have demonstrated significant potentiation of Bt activity by coadministering serine protease inhibitors. A variety of serine protease inhibitors isolated from plant and animal sources were effective at low levels in enhancing Bt activity against their target insects. The activity of HD-73 and HD-1 Btk proteins were potentiated by serine protease inhib-

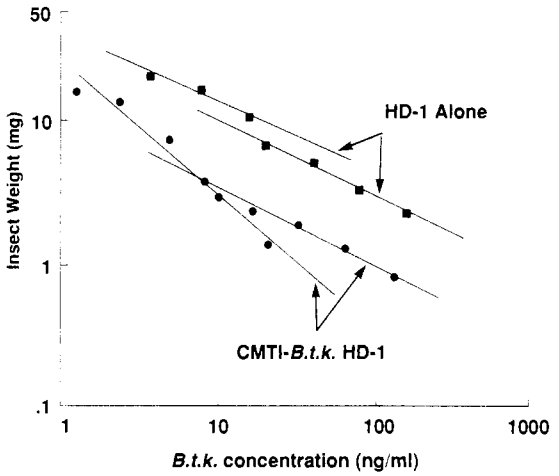

Figure 4. Activity of in planta expressed Btk HD-1 protein alone and CMTI–Btk HD-1 fusion protein. The tobacco plant extracts were prepared as described in Methods and Materials. Btk HD-1 protein concentrations of the plant extracts were determined by western analysis and were bioassayed in the larval growth reduction assay with tobacco budworm.

itors. This potentiation of Btk activity was observed for all four lepidopterans tested (Table IV). The soybean Bowman–Birk inhibitor did not enhance the activity of either the full-length Btk proteins, suggesting that this inhibitor may interfere with the activation of the full-length protein. The lack of potentiation of the HD-73 tryptic fragment is not understood since excellent potentiation was observed with the *T. ni* gut juice cleaved HD-73 protein and the tryptic fragment of HD-1, which was generated simultaneously with the HD-73 tryptic fragment.

Btt activity against the Colorado potato beetle was also potentiated with either soybean protease inhibitor. The activity of the mosquitocidal protein, Bti, was potentiated by the Kunitz inhibitor. However, the Bowman–Birk inhibitor was not effective in increasing Bti activity. This extends the utility of this potentiation to three distinct classes of Bt proteins.

Our primary objective was to enhance Bt activity in plants. Therefore, the ultimate demonstration of protease inhibitor potentiation of Bt activity was the coexpression of the two proteins in plants, which was accomplished by the expression of a CMTI-Btk HD-1 fusion protein. Although trypsin inhibitory activity could not be directly determined in the plants containing the fusion CMTI–Btk HD-1 protein, the fact remains that the single change in these plants was the presence of the CMTI protein. As predicted from the in vitro data, the protease inhibitor potentiated the Bt protein activity by approximately 6-fold (Figure 4).

The mechanism by which the protease inhibitors potentiate Bt insecticidal activity is unknown. Numerous groups have studied the deleterious effects of the protease inhibitors on insects. Much of this work centered on legume seeds that are insecticidal and contain high concentrations of serine protease inhibitors (1–4%) (Ryan, 1983). A specific variety of cowpea seed was selected for resistance to the larvae of the bruchid beetle, *Callosobruchus maculatus* (Gatehouse and Boulter, 1983). Biochemical studies by Gatehouse and Boulter (1983) suggested that the elevated levels of cowpea inhibitor within these seeds, double the amount found in susceptible cowpea varieties, was responsible for the insect resistance. These workers suggested that the antimetabolic activity of the cowpea inhibitor, the direct inhibition of larval protein digestion, was the mode of action of these trypsin inhibitors. This theory was recently refuted by Xavier-Filho et al. (1989) who found no correlation between the levels of protease inhibitors in cowpea seeds and resistance of cowpea seeds to insect attacks. However, the observation that protease inhibitors exhibit insecticidal activity was confirmed by cloning the inhibitor gene directly into plants (Hilder et al., 1987).

The levels of serine protease inhibitors (see Table I) that potentiate Bt activity are approximately 10^5 times below the insecticidal levels reported by Gatehouse and Boulter (1983). Furthermore, potentiation was observed with several insect species that possess very different gut juice protease compositions. Most lepidopterans contain high concentrations of trypsin-like gut proteases (Miller et al., 1974; Murdock et al., 1987), whereas the Colorado potato beetle gut proteases are primarily of the thiol type (Murdock et al., 1987). It is unlikely that the observed potentiation is due to direct inhibition of gut juice proteases. A related mechanism proposed by Broadway and Duffey (1986) is that the protease inhibitors induce the overproduction of gut juice proteases, which depletes sulfur-containing amino acids required for growth.

Other modes of action of the protease inhibitor enhancement of Bt may involve the inhibition of specific gut membrane associated proteases that serve to inactivate Bt or inhibition of proteases that are required for the activation of inactive but essential zymogens (e.g., chitin synthase). Leighton et al. (1981) have suggested that the chemical insecticide diflubenzuron (Dimlin) functions by inhibiting a chymotrypsin that may be required for activation of the inactive chitin synthase zymogen. Recent studies by Hofmann et al. (1988) suggest that insect gut membrane receptors are related to Btk specificity. Protease inhibitors may inhibit the degradation of membrane-bound receptors, therefore increasing their half-lives and the ability to bind Bt proteins. Since the mode of action of Bt itself has not been conclusively demonstrated, we can only speculate on how these inhibitors potentiate Bt activity.

Although their exact mode of action remains unknown, protease inhibitors do effectively potentiate Btk, Btt, and Bti activities. The ability to potentiate the efficacy of the commercial Dipel and Vectobac formulations emphasizes the immediate commercial application of these inhibitors. The ability to increase the insecticidal efficacy of Bt proteins expressed in genetically improved plants should have even more significant and long-term implications and benefits.

ABBREVIATIONS USED

Bacillus thuringiensis var. kurstaki, Btk; *Bacillus thuringiensis* var. tenebrionis, Btt; *Bacillus thuringiensis* var. israelensis; Bti; squash protease inhibitor (*Curcurbita maxima* trypsin inhibitor), CMTI; benzoyl-DL-arginine *p*-nitroanilide, BAPNA; dithiothreitol, DTT; sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SDS-PAGE.

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Protein Nutritive Value of a New Cultivar of Bean (*Phaseolus vulgaris* L.)

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The objective of this paper was to report the proximate composition, amino acid profile, and protein nutritive value of a new cultivar of dry bean (Carioca 80), which has been developed by crossing the Brazilian cultivar Carioca with the variety Cornell 49-242. In addition to improved productivity and resistance to rust and anthracnosis, the new cultivar presented methionine bioavailability of 57%, digestibility around 70%, and biological value from 75 to 80%. The limiting amino acid was methionine. Supplementation with 0.3% of methionine, on a protein basis, raised the biological value to 92%, superior to that of casein utilized as reference. The PER at 10% dietary protein did not differ from that of cultivars Carioca and Aeté 3 used in the PER assays for comparison, but at 21% bean protein the PER of Carioca 80 was 40% higher.

Dry beans are a very important source of calories and proteins for people in many countries. In Brazil legume seeds, excluding soybean and peanuts (FAO, 1966), contribute daily intake of 220 kcal and 14.8 g of protein. *Phaseolus* proteins have been characterized as having low nutritive value due to limiting amounts of sulfur-containing amino acids, low digestibility, low bioavailability of essential amino acids, presence of toxic proteins, and other antinutritive factors (Sgarbieri and Whitaker, 1982; Sgarbieri and Garruti, 1986; Sgarbieri et al., 1979; Durigan et al., 1987a,b). It has also been shown that whole beans and isolated bean proteins, both raw and heat-treated, stimulate considerably greater excretion by the rat of endogenous nitrogen, when compared with a casein or protein-free diet (Oliveira and Sgarbieri, 1986a,b).

In this paper we report a new dry bean cultivar with higher protein digestibility, high methionine bioavailability, and higher biological value than the Brazilian cultivars already studied.

MATERIALS AND METHODS

Cultivar. A new cultivar was developed at the Agronomic Institute of Campinas, SP, Brazil, named Carioca 80 resulting from crossing the cultivar Carioca with the variety Cornell 49-242. Plant selection was initiated with the F_2 generation and continued with the F_3 after artificial inoculation of the fungus responsible for anthracnosis. In this generation no segregations were observed for the gene *Are*; therefore, the progenies in F_4 were planted under field conditions (Pompeu, 1979). Further selection was performed in the F_5 generation for characteristics like resistance to the rust fungus and to the common mosaic virus, thus initiating the evaluation for productivity. The cultivar Carioca 80 resulted, finally, from three isogenic strains identified by the numbers 10-5-1, 10-6-2, and 10-9-1 (Pompeu, 1982). The new cultivar was 10–15% more productive than the cultivar Carioca, besides being resistant to the rust fungus, to anthracnosis, and to common mosaic virus. It also presented improved nutritional properties.

Cooking Conditions. Beans were soaked in distilled water at room temperature (12–48 h) and then cooked under pressure (15 psi, 40 min) in a pressure cooker. Cooked beans, with or without soaking water, were frozen and freeze-dried prior to grinding for use in the experiments.

Chemical and Biochemical Determinations. Total protein (% N × 6.25), total lipids, crude fiber, and ash contents were determined by AOAC (1980) procedures. The neutral detergent residue (NDR) was determined by the method of Van Soest and Wine (1967). The pepsin pancreatin residue was obtained by the procedure of Hellendoorn et al. (1975) using pepsin (2000 U/mg of protein) and pancreatin (350 FIP-U/g of protease; 7500 FIP-U/g of lipase; 7500 FIP-U/g of amylase) both from Sigma Chemical Co. Amino acids were determined by ion-exchange chromatography (Spackman et al., 1958) on a Beckman Model 119 CL amino analyzer (Beckman Instruments, 1977). Methionine was also determined by the sodium nitroprusside colorimetric reaction of a pepsin/pancreatin digest according to Tannembaum et al. (1969) as modified by Badiale (1979) and by the reaction of BrCN of a bean flour suspension followed by gas–liquid chromatography of the reaction byproduct CH_3SCN, according to Apostolatos and Hoff (1981). Trypsin inhibitor activity was measured in the raw and cooked bean extracts by the method of Kakade et al. (1969) with casein as enzyme substrate. Lectin activity (hemagglutination) was performed in raw and cooked bean extracts by the serial dilution procedure using

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