lesterol than dietary steroids in mammals, there has been intense interest in blocking cholesterol biosynthesis as a treatment for atherosclerosis, and many compounds have been assayed as mammalian hypolipidemic and hypocholesterolemic agents. These series of compounds may hold promise as insecticides which block terpene and therefore JH, ubiquinone, or pheromone biosynthesis in insects or which act by another mechanism possibly illustrated by the acute and chronic effects of the clofibrate analogues or precocene.

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Effect of Trypsin Inhibitors on Growth and Metamorphosis of Corn Borer Larvae *Ostrinia nubilalis* (Hübner)

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To determine if the naturally occurring proteolytic enzyme inhibitors of plants are related to resistance of food plants to insects, two purified inhibitors were added to the diets of young borer larvae, *Ostrinia nubilalis* (Hübner). Soybean trypsin inhibitor (Kunitz), incorporated at levels of 2–5% in the diet, inhibited growth of the larvae and delayed pupation, but did not prevent completion of the life cycle. The trypsin inhibitors of corn (maize) were prepared by a modified method and characterized with respect to amino acid composition and interaction with trypsin. The inhibitors of corn, which are weak inhibitors of trypsin, had no effect on growth or metamorphosis of the larvae. It is suggested that the proteolytic enzyme inhibitors of legumes, which are strong stoichiometric trypsin inhibitors, may be related to the resistance of these plants to the corn borer.

The proteolytic enzyme inhibitors of plants may be a mechanism of protection of plants against insect infestation. To study this possibility, we have chosen the European corn borer, *Ostrinia nubilalis* (Hübner), an insect that does extensive damage to corn (maize) and other vegetable crops, and two purified plant inhibitors, one from soybeans and one from corn. Soybean plants suffer little damage by the corn borer, whereas corn plants are this insect's principal host.

Previous investigations on soybean inhibitors with other insects have already provided support for this hypothesis.

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A few examples will be cited. Lipke et al. (1954) found a protein fraction of soybeans that inhibits growth and proteolytic activity in vitro of meal worm larvae, *Tribolium confusum*. Birk and Applebaum (1960) measured growth of *Tribolium castaneum* on partially purified soybean inhibitor fractions. Marked retardation of larval growth resulted from incorporation of fractions containing Bowman–Birk inhibitor (Birk, 1961) into the diet at a level of 5%. Commercial Kunitz inhibitor (Kunitz, 1946) retarded growth to a lesser extent at the same level.

Green and Ryan (1972) discovered that there was a rapid increase of proteinase inhibitor I content in potato and tomato plants when injury to the plant occurred. The inhibitor content of leaves and stems was low in undamaged plants and increased rapidly throughout the plant's tissues that were exposed to air, when Colorado potato beetles were allowed to feed on individual leaves, or when the leaves were damaged with a paper punch. Thus, a rise in inhibitor content after injury may be a defense mechanism of the plant.

Enzymes of the digestive tract of several insect larvae are inhibited by Kunitz soybean trypsin inhibitor. These include the midgut proteinases of the meal beetle, *Tenebrio molitor* (Applebaum et al., 1964), the trypsin- and chymotrypsin-like proteinases of the cabbage butterfly, *Pieris brassica* (Lecadet and Dedonder, 1966) and the trypsin-like enzyme of the tobacco hornworm, *Manduca sexta* (Miller et al., 1974). In all cases, in contrast to the stoichiometric inhibition of bovine trypsin, a large excess of inhibitor is required for partial inhibition.

The present study was conducted to compare the effects of two plant inhibitors on the growth and development of corn borer larvae. Kunitz soybean inhibitor, a protein of 22 000 molecular weight and known amino acid sequence (Koide and Ikenaka, 1973), is a strong stoichiometric inhibitor of trypsin and a good inhibitor of chymotrypsin (Kunitz, 1947). The inhibitor from corn (Hochstrasser et al., 1967; Chen and Mitchell, 1973) is a protein of 21 000 molecular weight, which dissociates into subunits (Hochstrasser et al., 1970). It is a weak, nonstoichiometric inhibitor of trypsin and a still weaker inhibitor of chymotrypsin (Chen and Mitchell, 1973).

In experiments preliminary to this work (Fox and Kassell, 1973, 1974), we have shown that the trypsin-like activity of crude corn borer midgut extract is partially inhibited by both soybean and corn inhibitors; as with bovine trypsin, soybean inhibitor is far more potent.

MATERIALS AND METHODS

Materials. Crystalline soybean trypsin inhibitor (Kunitz) was purchased from Sigma Chemical Co., St. Louis, Mo. Whole corn flour for the preparation of corn inhibitor (see below) was a gift from the Kellogg Company, Battle Creek, Mich. Bovine trypsin was purchased from Novo Enzyme Corp., Mamaroneck, N.Y.

Analytical Methods. Amino acid analyses were carried out in a Beckman-Spinco analyzer, Model 120B, modified according to Eick et al. (1974). Essentially the method of Moore and Stein (1963) was used. Acid hydrolysis by the method of Simpson et al. (1976) and lengthening the short column of the analyzer to 23 cm permitted the determination of tryptophan at the same time as the other amino acids.

Disc electrophoresis was carried out by the method of Davis (1964). The protein bands were stained with Coomassie blue R250 and scanned at 550 nm in a Gilford spectrophotometer, using the linear transport accessory. Slices of unstained gels, corresponding in position to each of the stained bands, were homogenized in 2 mL of 50 mM Tris–HCl buffer, pH 8.2. After centrifuging, the inhibitory activity of the supernatant against trypsin was determined by the method of Erlanger et al. (1961), as modified by Kassell and Chow (1966).

Insects. A colony of European corn borers, *Ostrinia nubilalis* (Hübner), was maintained in the laboratory, starting with eggs supplied through the courtesy of Drs. T. A. Brindley and W. D. Guthrie of the Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ankeny, Iowa. The culture method was that described by Guthrie et al. (1965 and 1971), adapted to a smaller scale, but with no significant changes in the procedures. The meridic diet of Guthrie et al. (1971) was used.

Two incubators, designed and built by Mr. Harold E. Eick of the Medical College of Wisconsin, were equipped with humidity controls, day and night temperature controls, and constant light. One, maintained at 26 °C for 14 h and 16 °C for 10 h daily, at 75% relative humidity, held the cages for emergence, mating, and egg laying. The other, at 26 °C constant temperature and 75% humidity, held the plastic diet dishes or vials in which the larvae were raised. The construction of the incubators will be described in detail separately (Eick and Kassell, 1977).

RESULTS

Purification and Characterization of Corn Inhibitor. The inhibitor was prepared from whole corn flour by the method of Chen and Mitchell (1973) except for two modifications: (1) The CM-cellulose chromatography preceded the Sephadex G-75 step as in the procedure of Hochstrasser et al. (1967). (2) Before the two chromatography steps, the inhibitor solution was heated as in the method of Melville and Ryan (1972) for the preparation of potato chymotrypsin inhibitor I. The solution was rapidly heated and maintained for 6 min at 80 °C, then cooled and centrifuged for 30 min at 39 000*g*. A heavy precipitate formed when the temperature reached 70 °C, but there was almost no loss of inhibiting activity from the supernatant solution.

From 1 kg of corn flour, 101 mg of product was obtained. From the disc electrophoresis scan (see below) this product was 90% inhibitors.

Attempts to improve the yield by using other starting materials were not successful. Corn germ (a gift from Krause Milling Co.) was a much poorer source of inhibitor; after the heat step, the solution contained only 20% as much inhibitory activity as the corn flour. Two by-products in the preparation of corn syrup, 60% corn gluten, and dried corn steep liquor concentrate (gifts from A. E. Staley Manufacturing Co. and from the Cargill Co.) were tested, but offered no advantage over the corn flour.

The purified product showed three protein bands on disc electrophoresis (Figure 1). The percentage of the total protein in each band, calculated from the areas of the peaks in the scan, was 63, 27, and 10% for bands 1, 2, and 3, respectively. The trypsin inhibitor assay on the extracts of the gel slices showed that bands 1 and 2 contained inhibitors in a ratio of 5 to 2, very close to the proportion of protein in these bands. No inhibitory activity was detected in the third band, a double band, which thus represents about 10% of protein impurities. Chen and Mitchell (1973) reported two inhibitors from sweet corn while Halim and Mitchell (1973) found three and four protein bands, respectively, by electrophoresis of the inhibitors of opaque-2 and floury-2 corns.

The amino acid composition of the inhibitor mixture (Table I) was calculated on the subunit molecular weight of 7000 reported by Hochstrasser et al. (1967). The values

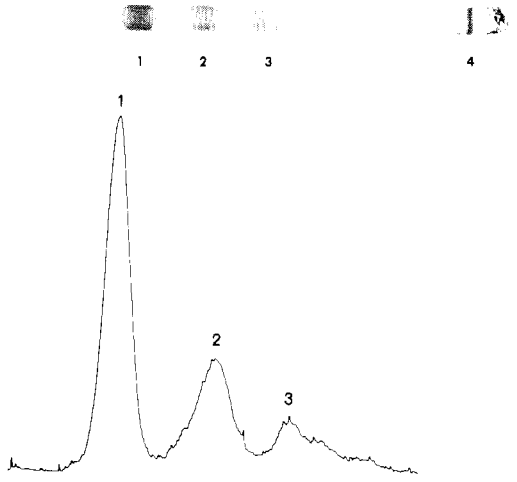

Figure 1. Disc gel electrophoresis with spectrophotometric scan of purified corn inhibitors. Band 4 is the tracking dye.

Table I. Amino Acid Composition of the Corn Inhibitor

Amino Acid	Residues per mole[a] Found	Whole no.	Hochstrasser (1970)
Tryptophan	1.02	1	0
Lysine	1.92	2	1
Histidine	1.14	1	1
Arginine	4.14	4	8
Aspartic acid[b]	4.02	4	3
Threonine	4.50	5	3
Serine	3.47	4	3
Glutamic acid[b]	7.11	7	5
Proline	6.62	7	10
Glycine	6.81	7	7
Alanine	5.36	5	4
Cystine	4.09	4	6
Valine	3.85	4	2
Methionine	0.83	1	1
Isoleucine	2.78	3	4
Leucine	5.10	5	6
Tyrosine	1.29	1	1
Phenylalanine	0.79	1	0
Calculated subunit weight		7027	

[a] Calculated to approximate the 7000 subunit molecular weight found by Hochstrasser (1970). [b] The values for aspartic and glutamic acids include asparagine and glutamine.

for most of the amino acid residues are close to whole numbers. A few (threonine, alanine, tyrosine, and phenylalanine), however, are not within experimental error of whole numbers. This is consistent with the expected composition of a mixture containing 90% of quite similar isoinhibitors in which a few amino acid substitutions have occurred. For this reason, and because this is the mixture of isoinhibitors consumed by the larvae under natural conditions, the inhibitor mixture was used without further separation.

The marked differences in composition from the inhibitor of Hochstrasser et al. (1970) may reflect a difference in the type of corn used to prepare meal or flour in Germany and the United States. Mitchell's group did not report data for amino acid composition. Comparison of the amino acid compositions of other plant inhibitors (Birk, 1976; Kassell and Williams, 1976) reveals amino acid substitutions among varieties and among the subunits of a single inhibitor.

A study of the reaction of the purified mixture with trypsin (Figure 2) demonstrated a weak nonstoichiometric inhibition. For 50% inhibition, an inhibitor–trypsin ratio of six is required, based on a molecular weight of 24 000 for trypsin (Cunningham et al., 1952) and 7000 for the inhibitor subunit. The interaction with trypsin is so far from stoichiometric that we cannot tell whether the complex is formed with the whole molecule or with a subunit. The results are similar to one of the two concentrations used by Chen and Mitchell (1973). For comparison, the inhibition curve of Kunitz soybean trypsin inhibitor, taken from the data of Coan and Travis (1971), is also shown in Figure 2.

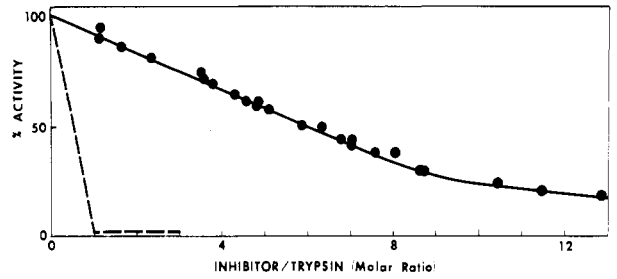

Figure 2. Inhibition of bovine trypsin by purified corn inhibitors and by Kunitz soybean inhibitor, with benzoyl-L-arginine-*p*-nitroanilide as substrate (Erlanger et al., 1961; Kassell and Chow, 1966). At 100% activity, for 14 μg of trypsin alone, there was a change in absorbance at 410 nm of 0.48 in 10 min. The soybean inhibitor curve is taken from the data of Coan and Travis (1971): (●——●) corn inhibitor, (- - - -) soybean inhibitor.

Growth and Survival of Larvae on Diets Containing Inhibitors. The experimental diets consisted of the diet of Guthrie et al. (1971) in which 2–5% of soybean inhibitor or 2–3% corn inhibitor was uniformly distributed. For the control diets, dextrose was added in equivalent weights to the inhibitors.

There were two series of growth experiments. In the first, eight larvae 5 to 8 days after hatching were weighed as a group and placed on a loosely packed layer of 16 g of diet in a ventilated petri dish. The dishes were placed in the 26 °C incubator. A few drops of water were added as needed to keep the diet moist. Weighings were repeated on the fourth day and then on alternate days for 12–16 days. Because of the difficulty in relating survival to diet, owing to the cannabalistic tendency of corn borer larvae (Beck and Stauffer, 1950), in the second series of experiments 8 or 12 larvae/treatment were grown individually in vials plugged with cotton (Beck and Stauffer, 1950) on 3 g of diet. After the initial weighing of the group, the larvae were weighed individually on a similar schedule. The two methods gave essentially the same relative growth for control and inhibitor-containing diets.

Figure 3, showing the growth curves for the individual groups, demonstrates the consistency of the results, which are evaluated in Table II. Larvae grown on diets containing at least 2% of soybean inhibitor (curves 9–12) gained significantly less weight than the controls. Increasing the level of inhibitor from 2 to 3% (curves 3–8), or even to 5% in two experiments (curves 1 and 2), did not enhance the growth retarding effect.

With the corn inhibitor there was no effect on growth at levels of 2 or 3% (Figure 3, curves 13 and 14). These experiments were not continued.

The percentage survival of the larvae grown in the individual vials was higher than on the plates (Table III); this is attributable to their not being damaged by other insects. The inhibitors did not affect survival significantly.

Effect of the Inhibitors on the Life Cycle. Incubation was continued in the vials until pupae formed. The larvae on soybean inhibitor diet (Figure 4b) and the

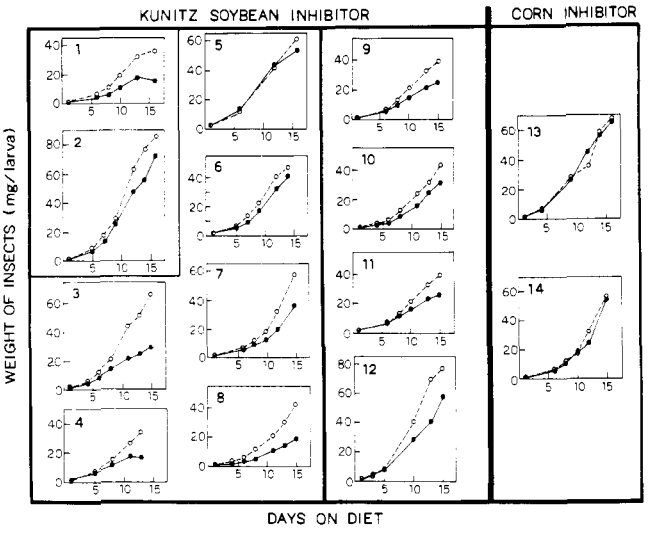

Figure 3. Growth of corn borer larvae on diets containing inhibitors. Level of soybean inhibitor: 1 and 2, 5%; 3–8, 3%; 9–12, 2%. Level of corn inhibitor: 13, 2%; 14, 3%: (O——O) control; (●——●) experimental.

Table II. Effect of Kunitz Soybean Trypsin Inhibitor on the Growth of Corn Borer Larvae[a]

Experiment no.[b]	Inhibitor in diet, %	Weight gain in 14–15 days	
		mg/larva	% of control
3	3	27.4	43.3
4	3	41.7	87.2
5	3	48.2	82.4
6	3	40.0	87.1
7	3	34.5	61.9
8	3	17.4	42.5
Av		34.9	67.4
9	2	24.7	64.5
10	2	31.1	73.2
11	2	24.2	63.2
12	2	55.9	74.3
Av		34.0	68.8
Overall av		34.5	68.0 ± 16.1[c]

[a] Data from Figure 1. [b] Eight larvae/experiment except for no. 7 which had 12. [c] $p < 0.01$.

controls (Figure 4c) showed a wide variation in the time of pupation. On soybean inhibitor diet pupation was delayed to an average of 30 days, compared to 24 days for the controls and 23 days for the corn inhibitor diet (Table III).

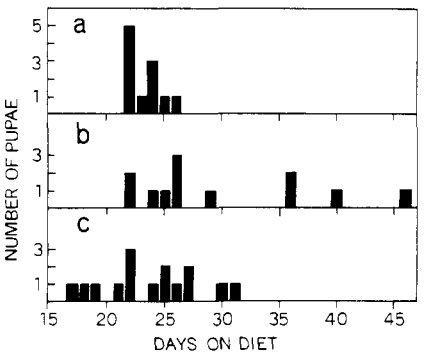

Figure 4. Formation of pupae on diets containing inhibitors: (a) 3% corn inhibitor, (b) 3% soybean inhibitor, (c) control.

Pupae were removed from the vials as they darkened; they were stripped of adhering silk and placed in an open petri dish. The petri dish rested on filter paper, moistened daily with water and sprinkled with sorbic acid crystals, inside a cylindrical wire mesh mating cage (Beck and Smissman, 1960). The cage was lined on the vertical surface with fine mesh screening and waxed paper was placed on the top. The cages were placed in the incubator with a day temperature of 26 °C and a night temperature of 16 °C. There was no significant difference among the three groups in the time between pupation and emergence (Table III).

When the moths emerged, the sides of the cages were sprayed lightly with water one–two times daily. Egg masses were cut out every few days from the waxed paper and incubated on moistened filter paper in petri dishes. We saw that the eggs were viable by allowing them to hatch, but no attempt was made to rear a second generation. The ratios recorded in Table III for adults emerged and egg masses per female are based on too few insects to be differentiated, but they show that all the groups went through their normal metamorphosis.

DISCUSSION

The thesis that resistance of plants to insects is related to the presence of digestive enzyme inhibitors is supported by the experiments with diets containing soybean inhibitors, but may be true only for leguminous and solanaceous plants, which contain relatively large quantities of powerful inhibitors. Gertler et al. (1967) estimated a content of 13.5 g of Kunitz inhibitor/kg of raw soybean meal. The results of the growth experiments on diets containing soybean inhibitor are similar to those of Birk and Applebaum (1960) in which Kunitz soybean inhibitor was added to the diet of *Tribolium castaneum*.

The results with soybean inhibitor prepared from the meal cannot be directly related to plant resistance, since

Table III. Life Cycle on Diets Containing Inhibitors

Diet	Method (see text)	Larval survival[a]		Pupae formed		Adults emerged		Egg masses per female
		No.	%	No.	Average day	No.	Average day	
Control	Plate	61/84	73					
Control	Vial	16/20	80	15/16	24	9/15	38	31/4 = 7.8
Soybean inhibitor								
5%	Plate	10/16	63					
3%	Plate	17/36	47					
3%	Vial	14/20	70	12/14	30	11/12	41	67/5 = 13.4
2%	Plate	23/32	72					
Corn inhibitor								
3%	Vial	11/12	92	11/11	23	6/11	37	13/3 = 4.3
2%	Plate	6/8	75					

[a] Larvae placed on diet 5–8 days after hatching (second to third instar). Survival after 15 days on diet for plate method; vials were kept up to 60 days.

these seeds are not consumed by the corn borer. However, the data of Green and Ryan (1971) and our own unpublished data (Fox and Kassell, 1977) for corn plants indicate that not only seeds, but also leaves and stems, contain inhibitors, although in smaller quantities. In the case of tomato and potato plants (Green and Ryan, 1971), the inhibitors of the stems and leaves were assayed immunologically, using antibody prepared against chymotrypsin I inhibitor from the potatoes (Ryan, 1967). This indicates the close similarity, if not identity, of the inhibitors in the various parts of the plant. Therefore, it is reasonable to assume that the parts of the soybean and corn plants consumed by the borer also contain the inhibitors.

Inhibition of growth on soybean inhibitor diets may indicate a relationship between the presence of proteinase inhibitors and avoidance of soybean fields by the corn borer. Although the growth inhibition and delay in pupation are not striking, small changes of this nature may be sufficient to tip the ecological balance in favor of the plant. The present experiments are the first of this nature with a purified inhibitor and a phytophagous insect that is a serious agricultural pest.

The failure of corn trypsin inhibitor to affect larval growth and metamorphosis may be interpreted in the light of the markedly different inhibiting potencies of soybean and corn inhibitors, as shown in Figure 2. Experiments with rats have shown similar differences between soybean and corn inhibitors. Although there is some disagreement in the literature, there is little doubt that part of the growth inhibiting effect of raw soybean meal is caused by trypsin inhibitors (Rackis, 1965; Kakade et al., 1973). In contrast, the corn trypsin inhibitor did not affect the growth of rats (Mitchell et al., 1976).

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