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**Structure and Function of Protease Inhibitors
from Legume Seeds and Cereal Grains
and Their Interaction with Digestive Enzymes
of Stored Product Insects**

Y. Birk, K.J. Kramer, S.W. Applebaum, G.R. Reeck

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Title

STRUCTURE AND FUNCTION OF PROTEASE INHIBITORS FROM LEGUME SEEDS AND
CEREAL GRAINS AND THEIR INTERACTION WITH DIGESTIVE ENZYMES OF STORED PRODUCT
INSECTS

Investigators' Names
(Principal listed first)

YEHUDITH BIRK

KARL J. KRAMER

SHALOM W. APPLEBAUM

GERALD R. REECK

Investigators' Institutions

THE HEBREW UNIVERSITY, REHOVOT

U.S.D.A.

THE HEBREW UNIVERSITY - REHOVOT

KANSAS STATE UNIVERSITY - MANHATTAN

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Yehuda Birk

Signature
Principal Investigator

Signature
Institution's Authorizing Official

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ABSTRACT

1. Numerous serine proteases with specificities of trypsin and chymotrypsin, as well as cysteinyl proteases, have been identified and separated from larval midguts of three major stored product insects pests: the red flour beetle Tribolium, the rice weevil Sitophilus and the Indianmeal moth Plodia. The proteases are inhibited by specific serine protease inhibitors and/or by compounds that selectively inhibit cysteinyl proteases. They are also inhibited by protein protease inhibitors isolated from a variety of seeds.
2. A labile protein from soybeans that inhibits selectively Tribolium proteases but does not inhibit trypsin or chymotrypsin, has been isolated and partially characterized.
3. A proteinaceous trypsin and chymotrypsin inhibitor that inhibits also Tribolium proteases has been isolated from amaranth seeds and characterized.
4. The sequence determination of CI, the trypsin-chymotrypsin inhibitor from chick peas is approaching completion.
5. An improved method for purification of corn trypsin inhibitor (CTI) in large quantities has been developed.
6. The gene for CTI has been cloned into yeast, E. coli and possibly into both alfalfa and tobacco plants.
7. α -Amylases have been purified from four species of stored product insects, including the rice weevil that has an exceptionally high content of amylase and a low level of proteases.
8. The presence of amylase inhibitors has been demonstrated for the first time in rice. Several of them selectively inhibit the rice weevil amylase and show no inhibition of human amylase.

In conclusion: The results of our collaborative research indicate the existence in seeds and grains of a wide spectrum of proteinaceous inhibitors of digestive enzymes of a variety of stored products insects. The species specificity of the inhibitors with respect to different insects and the data from simulation experiments provide basic information towards practical utilization of these natural defense mechanisms.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The objectives of this research have been to isolate, characterize and modify protein protease inhibitors from legume seeds and wheat grain, that specifically inhibit digestive enzymes of stored-product insects, and to study the interaction of the isolated inhibitors, native and modified, with Tribolium castaneum proteases, and possibly α -amylases, as a model. The significance of this research is in pinpointing and understanding, at the physiological and molecular level, naturally-occurring, built-in defense mechanisms of seeds against the attack of insects.

RESEARCH REPORT

BACKGROUND

The control of stored grain insects is a complex and difficult problem that has taxed a large number of investigators for many years. Although malathion, synergized pyrethrins and Bacillus thuringiensis are approved as grain protectants in the U.S., none of these has performed satisfactorily in recent years. Malathion is effective against most insect species, but it loses activity rapidly, especially when the moisture content and grain temperature are high. In addition, resistance to malathion is already acute and particularly widespread in red flour beetle and Indianmeal moth populations, while even the lesser grain borer has exhibited a measurable tolerance to this insecticide. Pyrethrins are more limited in their effectiveness and relatively expensive to use. The bacterium is generally inactive toward Coleoptera and resistance has developed in the Lepidoptera.

Thus, there is a need for alternative methods for the control of insects in harvested cereal grains. With the advent of genetic engineering and the prospect of enhancing gene expression or moving foreign genes into recipient plants, one new route to the solution of crop-pest problems is the genetic engineering of plants to

contain economically important resistance factors. There are several plant genes with products that attack sites of vulnerability in insects. These products include protease inhibitors and α -amylase inhibitors. The precise biological roles played by these plant proteins are still being defined, but it has been suggested that they confer benefit to the plant by reducing its vulnerability to herbivores and pathogens. Examples of particular plant proteins suggested to be defenses include chymotrypsin inhibitors in tomato which may serve as protection against insects, trypsin inhibitors in cowpeas, which may confer resistance to cowpea weevils and α -amylase inhibitors from wheat which may help to deter stored-product insect predation (reviewed: Ryan, C.A., 1979, in: *Herbivores, their Interaction with Secondary Metabolites*, D. Janzen & G. Rosenthal, eds., Academic Press pp. 599-618; Applebaum, S.W., 1985, in: *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, G.A. Kerkut & L.I. Gilbert, eds., Pergamon Press, pp. 279-311; Birk, Y., 1987, in: *Hydrolytic Enzymes*, A. Neuberger & K. Brocklehurst, eds., Elsevier, pp. 257-305). We have chosen to focus our attention first on protease inhibitors because insects widely used proteases for the processes of digestion and molting. As mentioned, plants have evolved large numbers of different protein protease inhibitors. We know very little about insect digestive enzymes or about the effects of plant protease inhibitors upon them. Few species of Coleoptera or Lepidoptera have been studied by modern biochemical techniques with the aim of characterizing the digestive proteases. Some of the major plant pests are beetles including stored product pests, such as red flour beetle and rice weevil. In this research project we have been studying the gut proteases of the red flour beetle and of several other stored-product insects, and their interaction with plant protease inhibitors in detail, in order to determine if genetic engineering of protease inhibitors has any potential for application in stored grain insect control. Attention also has been focused on insect gut amylases and their inhibitors.

A better understanding of such a seed defense mechanism could have important application in storing grains or in selecting and engineering crop species that will allow grain to be stored better.

EXPERIMENTAL AND RESULTS

Following is a comprehensive report on the operations conducted within the framework of the research project in Manhattan and Rehovot. The full scientific data and results have already been compiled and are in the process of preparation and submission for publication.

I. INSECT PROTEASES AND THEIR INHIBITORS

1. General characterization of insect proteases.

The nature of the proteases in midguts dissected from larvae of three stored grain insect pests: the red flour beetle, the rice weevil, and the Indianmeal moth has been studied. Midguts from all three species contained numerous proteinases that differ in mobilities in polyacrylamide gels containing gelatin as a substrate. The major gelatin digesting enzymes had apparent molecular weights of 17 KD and 20 KD (Tribolium), 30 KD (Sitophilus) and 20 KD (Plodia).

Caseinolytic activity of the red flour beetle and rice weevil midgut extracts was strongly inhibited by compounds that are selective for cysteinyl proteinases (e.g., p-chloromercuriphenyl sulfonic acid and epoxysuccinylleucylamide-4-guanidinobutane). The caseinolytic activities in these preparations were stimulated by thio reducing agents, such as dithiothreitol. In contrast, casein digesting activity of the Indian meal moth was inhibited primarily by specific inhibitors of serine proteases, such as diisopropylphosphofluoridate, and was not affected by thiol reducing agents. A trypsin-like proteinase is the major enzyme in Indianmeal moth midguts. The pH optimum for the moth proteases was pH 10 whereas that for the beetle proteases was pH 6.

The ability of the 12KD corn trypsin inhibitor to inhibit the trypsin activity in extracts of larvae of Tenebrio molitor (the yellow mealworm) has also been tested. Tryptic activity was measured using a synthetic substrate, N-benzoyl-arginine-p-nitroanilide. Complete inhibition of this activity by the purified 12KD corn inhibitor was observed. Thus, the 12KD corn protein can inhibit an insect serine protease.

2. Characterization of total proteolytic activity of Tribolium castaneum larval midguts.

a. On protein substrates. Tribolium castaneum proteases were extracted from midguts of last instar larvae (henceforth larval Midgut Enzyme Solution [LMES]). The total proteolytic activity of LMES was maximal at pH 7.0 when assayed on casein and at pH 4.0 when assayed on hemoglobin. The activity decreased upon storage in the cold or after freezing or freeze-drying, but was fully restored after addition of cysteine or dithiothreitol, due to the presence of cysteinyl protease(s).

b. On synthetic, specific, substrates. LMES readily hydrolyses the specific synthetic substrates tosyl-L-arginine-methyl-ester and N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) - for trypsin and N-acetyl-L-tyrosine ethyl ester and N-acetyl-DL-p-nitroanilide (ATPNA) - for chymotrypsin. Both activities were fully inhibited by the proteinaceous, double-headed trypsin-chymotrypsin inhibitors, the Bowman-Birk Inhibitor (BBI) from soybeans and CI from chickpeas. The two activities were also inhibited by the serine-enzyme inhibitor phenyl methyl sulfonyl fluoride (PMSF). Tosyl-L-lysine chloromethyl ketone and Tosyl-L-phenylalanine chloromethyl ketone, which are specific active-site titrants for trypsin and chymotrypsin, respectively, showed full inhibition of the respective activities. The effects of various inhibitors on Tribolium larval midgut proteases, when assayed on protein and synthetic substrates, are summarized in Table 1. LMES proteases were separated by polyacrylamide gel electrophoresis and

TABLE 1. EFFECT OF INHIBITORS ON TRIBOLIUM LARVAL MIDGUT PROTEASES
WHEN ASSAYED ON DIFFERENT SUBSTRATES IN REACTION MIXTURE (RM)

Enzyme ¹ µg in RM	Substrate	PMS ⁶		FOS ⁷		TLCK ⁸		TPCK ⁹		PABA ¹⁰		FBA ¹¹		E-64 ¹²		BBI ¹³		CI ¹⁴		STT ¹⁵	
		Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %	Conc. RM	Inhib. %
0.1	CASEIN	8.0	41	1.28	92	0.5	100	0.5	94	3.0	65	12.5	79	1.2x10 ⁻³	100	50	0	20	0	20	0
0.1	HEMOGLOBIN	8.0	50	1.28	100	0.5	100	0.5	100	3.0	—	12.5	45	10 ⁻³	100	25	0	—	—	25	0
0.2	TYR ²	3.0	100	5.4	67	7.5	100	7.5	15	1.0	50	—	—	1.0	0	0.1	50	0.15	50	0.2	50
0.1	ATFE ³	0.75	100	6.8	97	7.5	25	7.5	76	—	—	7.0	50	—	—	0.075	50	0.1	50	1.5	50
0.1	BAPNA ⁴	3.0	100	1.0	4	0.7	70	0.7	14	0.2	58	90.0	42	1.0	10	0.2	80	0.1	50	1.0	80
0.1	ATPA ⁵	—	—	—	—	—	—	0.7	85	—	—	—	—	—	—	0.5	76	1.0	87	1.0	50

¹ "Equivalent" to bovine trypsin or chymotrypsin

² p-tosyl-L-arginine methyl ester

³ N-acetyl-L-tyrosine ethyl ester

⁴ N-benzoyl-L-arginine-p-nitroanilide

⁵ Acetyl-L-tyrosine-p-nitroanilide

⁶ Phenylmethylsulfonyl fluoride

⁷ Chloromercuribenzoate

⁸ N-o-tosyl-L-lysine chloromethyl ketone

⁹ N-o-tosylphenylalanine chloromethyl ketone

¹⁰ p-aminobenzamide

¹¹ Phenylbutylamine

¹² L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane

¹³ Bowman-Birk inhibitor

¹⁴ Quick-pee inhibitor

¹⁵ Soybean trypsin inhibitor

identified by staining the gels with solutions of the specific amidolytic substrates BAPNA and ATPNA. Three red distinct bands, one for trypsin-like activity and two - for chymotrypsin-like activity have been revealed. The separated, active, enzymes could be easily extracted from non-stained gels.

3. Separation and further characterization of Tribolium proteases and their interactions with inhibitors.

Most of the larval midgut proteolytic activity could be separated from the bulk of accompanying proteins by column chromatography on DEAE-cellulose. This resulted in a 20-fold purification and in a 100% yield, suggesting the possibility of removal of endogenous inhibitors. (Attempts to separate the proteases in a similar manner from whole larvae resulted in a very small yield and were not pursued). The proteases in the effluents comprised of three major populations: (a) with chymotrypsin-like specificity on the synthetic substrate ATPNA, lacking caseinolytic activity; (b) with trypsin-like specificity on the synthetic substrate BAPNA also hydrolysing casein; and (c) with caseinolytic activity only. They could be fully inhibited by inhibitors of cysteinyl proteases, such as p-chloromercuribenzoate and E-64 and only partly - by PMSF - the inhibitor of serine proteases. The essentiality of free thiol groups for enzyme activity and possibly also for active enzyme conformation was substantiated by the activating effect of thiol reducing agents (such as mercaptoethanol) on enzyme activity in solution and on freeze-dried enzyme preparations. A sharper separation between the different proteases, showing at least 3 cysteinyl proteases, was revealed by submission of the DEAE-cellulose active effluent to polyacrylamide gel electrophoresis (PAGE) including gelatin as substrates, and in parallel to gels including also the inhibitor E64, that inhibits cysteinyl proteases (Figure 1).

FIGURE 1. LOCATION OF PROTEOLYTIC ACTIVITY ON PAGE CONTAINING 10% ACRYLAMIDE AND 0.2% GELATIN.

Running with Tris-glycine buffer (0.1M, pH 8.9) for 1 h at 200 V, 40 mA. After running, the gel was incubated for 30 minutes in tris-HCl buffer (0.1 M, pH 7.8) at room temperature and stained with coomassie blue. Proteolytic activity is indicated by clear areas on the blue background.

(a) and (b): Active samples from affinity chromatography on a PABA-Agarose column.

(a) Gel incubated in Tris-HCl buffer (0.1 M, pH 7.8).

(b) Gel incubated in Tris-HCl buffer (0.1 M, pH 7.8) containing 0.01 M DTT.

(1) is the non-bound effluent from the PABA-agarose column. It is active only after incubation with DTT. It displays cysteinyl-protease activity, and does not show trypsin-like activity.

(2) comprises of the trypsin-like activity, bound to the PABA-agarose column and released by 6 M urea. It is not influenced by DTT.

(c) wells 1,2,3 - Contain 10 μ g samples of LMES

wells 4,5 - Contained 10 μ g LMES samples as above plus 1 nm of E.64 - the specific inhibitor of cysteinyl proteases.

The PAGE-gelatin technique was further employed for the study of the effect of naturally-occurring proteinaceous protease inhibitors. Whereas BBI, molecular weight 8KD, (the Bowman-Birk trypsin-chymotrypsin inhibitor from soybeans) inhibited the trypsin- and chymotrypsin-like activities on low molecular weight synthetic substrates, it did not affect the caseinolytic activity, when assayed in solution. However, when incorporated to PAGE-gelatin assays, it strongly affected proteolysis.

This last finding led to the isolation of the hitherto unknown *Tribolium* trypsin-like enzyme by affinity chromatography on a p-aminobenzamidine (PABA)-agarose column. The affinity-bound protease (P_1) was released from the column by a solution of 6M urea. P_1 was fully active on the specific substrate BAPNA and showed a single band of 21 KD on silver stained PAGE-SDS. Affinity chromatography for cysteinyl proteases was then performed on the early effluent that did not bind to the PABA-agarose. The latter was mounted on a TS-6B thiopropyl sepharose column and the cysteinyl proteases were released by buffered cysteine, revealing 2 cysteine proteases of 30 KD and 34 KD on PAGE-SDS.

Attempts to achieve the isolation of P_1 by reversed phase or ion exchange HPLC (on Vydac 218 or Mono Q columns, respectively) resulted in poorer purification and partial loss of activity.

4. Inhibitors of *Tribolium* proteases.

In view of the above findings on the presence of trypsin- and chymotrypsin-like enzymes in *Tribolium castaneum* LMES, the study of specific *Tribolium* protease inhibitors, that do not inhibit trypsin or chymotrypsin, has been extended also to several trypsin and chymotrypsin inhibitors, and they will be described in the forthcoming sections 5 - 8.

The isolation and characterization of the specific inhibitor of *Tribolium* proteases from soybeans has been rather problematic. The protein fraction in soybeans, that inhibits *Tribolium* proteases but does not inhibit trypsin and chymotrypsin, has been extremely

sensitive to the common isolation procedures. We assume that this protein fraction is a potent inhibitor of *Tribolium* proteases as long as it is in the native assembly in the soybean meal. The initial aqueous extract of the soybean still contains a major part of accompanying proteins and other compounds which presumably provide a protective environment for the conformationally labile inhibitor fraction. In spite of the fact that the *Tribolium*-inhibitory potency decreases with the steps of purification, we were able to separate a specific inhibitory fraction by means of ammonium sulphate fractionation followed by ion exchange chromatography on carboxy methyl cellulose, combined with affinity chromatography on sepharose-trypsin, in order to separate between the specific *Tribolium* protease inhibitor and the soybean trypsin inhibitors. The resulting active inhibitor fraction had an amino acid composition (in nanomols) of: Asp 36; Glu 78; Ser 22; Gly 122; His 63; Arg 33; Thr 70; Ala 75; Pro 49; Tyr 19 Val 34; Met 22; 1/2 Cys - traces; Ile 16; Leu 20; Phe 8; Lys 47. It showed two major bands (~20 KD and ~30 KD) in polyacrylamide gel electrophoresis. This inhibitory fraction differs from the common legume seed protease inhibitors in the lack of S-S bonds, a difference which may account for its conformational lability during the steps of purification, including freeze-drying.

5. Isolation and characterization of a trypsin-chymotrypsin inhibitor from amaranth seeds that inhibits also *Tribolium* proteases.

Amaranth seeds serve as a rich nutritional source, mainly in the Third World, South American and Eastern Asian countries. Research published during the last years emphasizes the high protein content found in these seeds. The protease inhibitor(s) of amaranth seeds were first reported in 1985. However, the physiological and biochemical features were not studied.

The amaranth inhibitor (AM.I) has been extracted by buffer elution of amaranth seed meal. It inhibits trypsin, chymotrypsin

and *Tribolium* midgut proteases. Further purification was carried out by affinity chromatography on trypsin-sepharose (AM.I_{AFF}) or by reversed phase HPLC (AM.I_{HPLC}). The inhibitor appeared as a single band on silver-stained SDS polyacrylamide gels, thus illustrating its purity. The molecular weight was 7KD, both on SDS polyacrylamide gels and in a G-50 Sephadex column. AM.I has been found to be stable at neutral and alkaline pHs for at least 2 hours of incubation. It is stable for at least 90 min at 37°C and is found stable to heat treatment of 40°C - 80°C for 15 min.

Further characterization of AM.I consisted of the following: Isoelectric point was determined using Dedopplkamkammer Dunnschicht isoelectrisch fokussing apparatus from DESAGA, on Serva Feinbiochem membranes, at a pH range 3 - 10 for 5 hours. The isoelectric point for AM.I was in the cationic range 7.9. The cationic nature and homogeneity of AM.I have also been confirmed by electrophoresis in cellulose acetate membranes at pH 6.9.

Purified AM.I was determined to have a specific extinction coefficient of 0.75 at 280 nm, and was found to be free of carbohydrate by periodic acid schiff gel staining.

The amino acid composition of AM.I_{HPLC} and AM.I_{AFF} is given in Table 2. It seems that the virgin inhibitor is AM.I_{HPLC}, whereas AM.I_{AFF} is presumably a breakdown product of the procedure that retained the inhibitory activity. The sequence determination, which is now in progress, will provide evidence whether this assumption is correct.

6. Improved method for purification of corn trypsin inhibitor (CTI).

Although we have been working with CTI for several years, until now we have not been able to purify large enough quantities for insect feeding studies and other experiments. Previously we used a procedure employing a combination of affinity chromatography using trypsin as the immobilized ligand and reversed phase HPLC to purify milligram amounts of CTI. However, the virgin inhibitor

TABLE 2. AMINO ACID COMPOSITION OF AMARANTH PROTEASE INHIBITORS ISOLATED BY REVERSE PHASE HPLC (AM.I_{HPLC}) OR BY AFFINITY CHROMATOGRAPHY ON A TRYPSIN-SEPHAROSE COLUMN

<u>Amino Acid</u>	<u>AM.I_{HPLC}</u>	<u>AM.I_{AFF}</u>
Asp/Asn	3	3
Thr	3	2
Ser	2	2
Glu/Gln	13-15	4
Pro	2-3	2
Gly	6	3
Ala	3	2
1/2 Cys	2-4	0-1
Val	4-5	4
Met	1-2	0
Ile	2-3	1
Leu	2	1
Tyr	1	1
Phe	1	1
His	1-2	1
Lys	2-4	0-1
Arg	5	2-3
Trp	1-2	4
TOTAL	54-66	33-36

was cleaved into two chains by this procedure. Recently, we have found that heat treatment and ammonium sulfate fractionation can yield material that is approximately 80% homogeneous. Hundreds of milligrams of nearly homogeneous virgin inhibitor can now be obtained (Figure 2).

7. Cloning and expression of the CTI gene.

We have obtained a cDNA clone that encodes CTI and a manuscript describing that work has been submitted for publication (Wen *et al.*, submitted). We have also used the plant gene vector Agrobacterium tumefaciens and plant tissue culture to introduce a cDNA clone of the CTI gene into both alfalfa and tobacco plants. The cloned CTI gene with protein signal peptide was subcloned into the binary vector pMON410A under control of the CaMV 35S plant promoter. Another clone lacking the signal peptide was produced

FIGURE 2. SIMPLIFIED PURIFICATION OF THE 12KD CORN INHIBITOR

The arrow indicates the 12K corn inhibitor. Samples for electrophoresis were:

1. Molecular weight markers
2. H₂O extract of corn meal
3. Boiled extract
4. Ammonium sulfate precipitate (0-20% saturation) of the boiled extract
5. Ammonium sulfate precipitate (20-40% saturation) of the boiled extract
6. Samples 5 and 4 combined

using oligonucleotide site directed mutagenesis and subcloned similarly to the previous clone. The vector plasmid used encodes a kanamycin resistant gene and nopaline synthase gene for plant expression. The two constructs were introduced into *A. tumefaciens* by triparental bacterial conjugation. Tobacco leaf discs and alfalfa petioles were infected with the *A. tumefaciens* containing the CTI genes. Nopaline was detected in a few tobacco plants and alfalfa calli, indicating successful gene transfer. Alfalfa embryos are now germinating. Nopaline-positive plants should produce CTI.

We have also obtained expression of the CTI gene in yeast. A construct was prepared in the yeast plasmid YEp13 that includes the yeast triosephosphate isomerase promoter and terminator gene sequences, the portion of the yeast α -factor gene that encodes the signal sequence of the prepro-form of that protein, and the CTI gene. Several transformed isolates were identified that secreted immunoreactive material, whereas no immunoreactive material was detected in the medium of a control yeast culture. CTI was

isolated by reversed phase HPLC from the culture medium of cloned transformed cells. It was highly effective in inhibiting trypsin.

8. Sequence determination of chick peas trypsin-chymotrypsin inhibitor (CI).

Sequencing of CI, the trypsin-chymotrypsin inhibitor from chick peas (8 KD) is advancing completion in a collaborative effort with Dr. W.J. Kohr in California. Following is the sequence of 32 amino acids, from the amino terminal end:

1				5					10
Gly.	Val.	Gly.	Tyr.	Lys.	Val.	Lys.	Ser.	Thr.	Thr.
			15					20	
Ala.	Cys.	Cys.	Asp.	Ser.	Cys.	Val.	(Cys).	Thr.	Lys.
									(?)
									(Ser)
		25						30	32
(Ile).	Pro.	Pro.	Gln.	Cys	(?).	(?).	Asn.	(Asp).	Met
					(Arg)	(Cys)			

II. INSECT AMYLASES AND THEIR INHIBITORS

1. Purification and characterization of amylases from stored produce insects.

α -Amylases were purified from four species of stored product insects including the rice weevil (two isozymes), red flour beetle (two isozymes), confused flour beetle (two isozymes) and yellow mealworm (one isozyme). The amino acid compositions, molecular weights, extinction coefficients, K_m values and V_{max} values for all the isoamylases were similar. Wheat amylase inhibitors inhibited all of the isozymes although K_i values varied with each enzyme. The bifunctional CTI inhibited α -amylases purified from the red flour beetle, confused flour beetle and yellow mealworm, but not those from the rice weevil, as well as α -amylases from human saliva or porcine pancreas.

2. Purification and cloning of α -amylase inhibitors and/or promoters from wheat and rice.

Wheat flour was extracted with hot 0.15 M NaCl and separated into four fractions by ammonium sulfate precipitation. Each fraction had a distinct polypeptide composition (>10 polypeptides as revealed by HPLC) and unique inhibitory profiles against human, porcine and insect α -amylases. Recombination of certain polypeptides resulted in a synergistic effect on the inhibitory activity. This result suggested that HPLC resolves the multimeric inhibitors into monomers. Two of the inhibitors which specifically inhibit insect α -amylases were purified by reversed phase HPLC and sequenced in a collaborative effort with Dr. Michael Richardson in England. The amino acid sequences are currently being used to identify genes which encode for the inhibitors. A third inhibitor specific for insect α -amylase but differing in relative electrophoretic mobility in nondenaturing polyacrylamide gels from the other inhibitors has also been purified.

Rice flour was also extracted with hot 0.15 M NaCl and fractionated by ammonium sulfate precipitation. α -Amylase inhibitory activity was present in the 0-40% fraction, whereas rice weevil protease inhibitory activity was found in the 40-65% fraction. After separation of the 0-40% fraction by HPLC, several peaks were found to inhibit human or porcine α -amylase, but only one specifically inhibited insect α -amylase (Fraction 7, Table 3). The amino acid composition of each fraction was determined (Table 4). A rice low molecular weight globulin cDNA clone (LMWG.L1) that probably encodes one of the inhibitory fractions was obtained and sequenced. All of the rice albumin fractions obtained by HPLC have remarkably similar amino acid composition. In addition, the amino acid composition deduced from the nucleotide sequence of clone LMWG.L1 reveals that its gene product is also a member of the same protein family. A rice 10 KD prolamin genomic DNA was also isolated and sequenced. Its upstream region (800 bp) which may contain the promoter sequence was subcloned into vector pPUR. Such

TABLE 3. ASSAYS OF INHIBITORY ACTIVITIES OF RICE RP-HPLC FRACTIONS

SOURCES OF AMYLASES	RP-HPLC PEAKS OF 0-40% (NH ₄) ₂ SO ₄ RICE ALBUMINS								
	1	2	3	4	5	6	7	8	9
Rice weevil 1	-	+++	+	+	-	-	++	-	-
Human salivary	-	+	+++	+++	-	-	-	-	-
Porcine pancreas	-	+++	-	-	-	-	-	-	-
<u>Bacillus</u> bacterial	-	-	-	-	-	-	-	-	-

One amylase unit was used in each assay and about 5 µg inhibitor protein were used in each assay.

TABLE 4. AMINO ACID COMPOSITIONS OF RICE ALBUMIN HPLC FRACTIONS AND CLONE LMWG.L1

AMINO ACIDS	RP-HPLC FRACTIONS OF RICE ALBUMIN								
	1	2	3	4	6	7	8	9	LMWG.L1
Asx	7.2	8.6	7.0	7.8	7.3	7.2	6.3	6.2	6.87
Glx	7.6	8.2	8.1	8.3	9.2	9.0	9.3	9.7	6.10
Ser	4.5	4.0	3.7	3.8	3.3	3.4	4.8	4.7	3.82
Gly	12.6	13.4	14.0	13.9	12.2	12.8	13.0	12.3	12.98
His	4.3	3.7	2.9	3.8	3.1	3.0	3.8	3.2	3.05
Arg	11.4	12.3	12.1	11.5	12.3	11.6	12.5	11.8	13.74
Thr	4.2	3.6	3.7	3.0	3.0	3.3	4.1	4.0	4.58
Ala	9.7	9.2	9.9	10.2	9.9	9.8	11.3	11.2	9.16
Pro	7.2	7.2	8.0	7.8	7.7	8.1	9.1	8.8	6.87
Tyr	3.6	3.9	3.2	3.5	3.9	3.5	3.5	3.3	3.82
Val	6.3	6.4	6.2	6.3	6.0	6.2	5.3	5.1	6.87
Met	1.9	1.9	2.1	1.6	1.5	1.8	1.3	1.2	3.05
Cys	8.5	8.3	9.4	8.2	9.6	9.6	6.7	7.9	7.63
Ile	1.3	0.9	0.7	1.2	2.6	2.2	1.4	2.4	1.53
Leu	5.8	5.8	7.2	6.9	6.4	6.4	6.2	7.2	6.11
Phe	1.6	1.3	1.2	1.1	1.3	1.3	1.2	1.0	1.53
Lys	2.3	1.3	0.8	1.2	0.6	0.6	0.2	0.1	0.76
Trp									1.53

a rice endosperm specific promotor may be useful for expression of foreign genes, such as those of amylase and protease inhibitors in transformed rice.

III. BIOASSAY OF INHIBITORS

To date we have had little success in inhibiting stored product insect growth in bioassays of cereal inhibitory fractions using a semi-artificial diet and three insect species, the rice weevil, red flour beetle and yellow mealworm (Baker and Mabie 1973, J. Econ. Entomol. 66,681). Apparently, the nutritional quality of the Baker diet is too high for revealing deleterious effects on insect growth by digestive enzyme inhibitors (Broadway and Duffey 1988, J. Insect. Physiol. 34, 1111). Also we have been severely limited in the amounts of potential inhibitory fractions that we can test (<0.1% wt/wt). To overcome the bioassay problems, we have developed less nutritious meridic diets that are depleted of protein (casein) but nevertheless support stored produce insect development quite well (rice weevil, red flour beetle, yellow mealworm and Indianmeal moth). We are currently retesting the cereal fractions for insect growth regulating activity using the protein-deficient semi-artificial diets.

COOPERATION

The first year's cooperation started with a brief visit of Jerry Reeck to Rehovot. This meeting, devoted mainly to planning and exchanging information and views, was followed-up by correspondence. In November 1987, Jerry Reeck visited the Rehovot group. Results were compared and discussed and consequently, short- and long-term experiments were jointly planned. In March 1989 Patricia Smirnoff from the Rehovot group spent a fruitful month with the Manhattan group. During that period methodologies were compared, experimental procedures were exchanged and experiments were performed jointly. In addition, there has been

a steady exchange of information via BITNET and a sharing of purified inhibitor preparations. For instance, Manhattan has sent CTI, the corn trypsin inhibitor to Rehovot and prepared a chickpea cDNA library; proteases and inhibitors isolated in Rehovot have been were characterized and assayed comparatively during Patricia Smirnoff's stay in Manhattan.

EVALUATION OF RESEARCH ACHIEVEMENTS

Midguts from larvae of three major stored grain insect pests (the red flour beetle Tribolium, the rice weevil Sitophilus and the Indianmeal moth Plodia contain numerous proteases that are strongly inhibited by specific inhibitors of serine proteases and/or by compounds that are selective for cysteinyl proteases.

Trypsin-like, chymotrypsin-like and cysteinyl proteases were identified and separated from the midguts of Tribolium castaneum larvae and characterized. all of them can be inhibited by protein protease inhibitors isolated from a variety of seeds.

The finding that BBI, the proteinaceous trypsin-chymotrypsin inhibitor from soybeans, strongly inhibitors Tribolium midgut proteolytic activity when the protein substrate was incorporated into polyacrylamide gel, but not in solution, led to the assumption that there is better mutual accessibility between the inhibitor and protease in the gel matrix. This may simulate the in vivo interaction of insect and raw soybean meal and bear relevance to the built-in defense mechanism in soybeans via insect-inhibiting components.

α -Amylases have been purified from four species of stored product insects, including the rice weevil that has an exceptionally high content of amylase and a low level of proteases. The presence of amylase inhibitors has been demonstrated for the first time in rice. Several of them selectively inhibit the rice weevil amylase and show no inhibition of human amylase.

Purification and cloning of α -amylase inhibitors and/or promoters from wheat and rice have been achieved.

The results of our collaborative research indicate the existence in seeds and grains of a wide spectrum of proteinaceous inhibitors of digestive enzymes of a variety of stored products insects. The species specificity of the inhibitors with respect to different insects and the data from simulation experiments provide basic information towards practical utilization of these natural defense mechanisms.

PUBLICATIONS

Following is a List of Publications, submitted and in preparation, resulting from the research project:

- Wen, L., Huang, J.K., Zen, K.C., Johnson, B.H., Muthukrishnan, S. and Reeck, G.R. (1990):
A cDNA CLONE THAT ENCODES THE CORN INHIBITOR OF TRYPSIN AND ACTIVATED HAGEMAN FACTOR (submitted).
- Johnson, D.E., Brookhart, G.L., Kramer, K.J., Barnett, B.D. and McGaughey, W.H. (1990):
RESISTANCE TO BACILLUS THURINGIENSIS BY THE INDIANMEAL MOTH, PLODIA INTERPUNCTELLA: COMPARISON OF MIDGUT PROTEINASES FROM SUSCEPTIBLE AND RESISTANT LARVAE (submitted).
- Yona, N., Applebaum, S.W. and Birk, Y. (1990):
ISOLATION AND CHARACTERIZATION OF SERINE AND CYSTEINYL PROTEASES FROM MIDGUTS OF TRIBOLIUM CASTANEUM LARVAE (in preparation).
- Tamir, S., Smirnoff, P. and Birk, Y. (1990):
ISOLATION AND CHARACTERIZATION OF A TRYPSIN-CHYMOTRYPSIN INHIBITOR FROM AMARANTH SEEDS THAT INHIBITS ALSO TRIBOLIUM PROTEASES (in preparation).
- Kohr, W.J., Smirnoff, P. and Birk, Y. (1990):
SEQUENCE DETERMINATION OF CHICK PEAS TRYPSIN-CHYMOTRYPSIN INHIBITOR (CI) AND OF ITS ACTIVE CNB₁ FRAGMENTS (in preparation).