Unidentified Toxins May Be Responsible for Soybean's Freedom from Insect Damage

GROWTH INHIBITORS

Effect of Soybean Inhibitors on Growth Of *Tribolium Confusum*

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The relatively large quantities of material needed for nutritional studies with vertebrates have prevented a comprehensive study of the toxicity of soybean inhibitors. This paper describes such a study using an insect whose responses to raw and heated soybean diets were similar to that of rats and chicks. The inhibitors were fed at levels comparable to those occurring in raw soybeans. No significant decrease in the growth rate was observed after the addition of the following inhibitors to the diets: soyin 2.5%, crystalline trypsin inhibitor 2%, alkaline, acid, or ethanolic extracts of soybean 5%. The material precipitable from a pH 4.6 extract of raw soybean by 0.475 saturation with ammonium sulfate was toxic to all the larvae at the 2.5% level, and inhibited the digestion of protein in vitro by 50%. Crude trypsin, methionine, amino acid mixtures, and high quality protein did not improve raw soybean nutritionally, but 2% yeast stimulated growth. Some of the toxicity of raw soybeans to vertebrates and the relative freedom of soybeans from insect damage may be due to the presence of a hitherto undescribed toxin in this legume.

THE INABILITY OF CERTAIN PESTS of stored grains to develop normally on soybean products was first reported by Mickel and Standish (*24*) and subsequently verified by other investigators (*8*, *11*, *12*, *23*). Because considerable interest exists in the nutritive value of soybeans for higher animals as well as for insects, the nature of the phenomenon involved in these scattered reports has been studied. The small amounts of food needed for the complete development of one of these pests of stored products, *Tribolium confusum*, has made it possible to determine the toxicity of known soybean inhibitors to this organism by inclusion of these substances in the larval diets at levels comparable to those occurring in raw soybeans.

Although the use of this insect has advantages for certain types of nutritional research, interpretation of the results of such investigations is limited by the absence of information concerning the relative digestibility of different rations, in this instance raw and autoclaved soybean meal. The larvae of *Tribolium* live in the food proper, and digestibility cannot be determined under such rearing conditions, since food and feces, both of which have a dry, mealy consistency, cannot be quantitatively separated. For this reason some other index of the extent of utilization of dietary protein was necessary for *Tribolium*. This paper, therefore, includes an in vivo study of the antitryptic activity of soybean inhibitors for the proteolytic enzymes of *Tribolium* and for vertebrate trypsin.

That supplements of methionine stimulated the growth of rats and chicks reared on diets containing raw soybean meal was first shown by Hayward *et al.* (*15*). The numerous studies that demonstrated that this effect was due to unavailability of absorbed methionine, rather than to a deficiency in this amino acid, have been reviewed by Mitchell (*25*) and Liener (*21*).

The ability of soluble fractions of raw soybean to inhibit trypsin was reported by Bowman (*5*) and by Ham and Sandstedt (*14*). Shortly thereafter, an inhibitor of this enzyme was isolated from raw soybean in crystalline form by Kunitz (*18*). Further evidence in favor of the participation of antitrypsins in the raw soybean effect was obtained by Liener (*20*) working with rats, and by Almquist and Merritt (*1*) with chicks, who showed that the addition of trypsin exerted a beneficial effect on the growth of animals fed raw soybean meal. Borchers and Ackerson (*4*) also ascribed a therapeutic action to crude trypsin powder, but reported that autoclaved trypsin powder or a 60% ethyl alcohol–soluble fraction of this material, presumably devoid of proteolytic activity, could replace the crude trypsin.

Liener (*20*) found that only half of the growth retardation induced by the presence of raw soybean meal in the ration could be counteracted by trypsin, and ascribed the remaining toxicity to the presence of a hemagglutinating agent in this legume. This material, which was named soyin, was isolated in a high state of purity as indicated by its physical properties (*26*), and exerted a depressing effect on both weight gain and food intake when added to the diet of rats.

Table I. Effect of Methionine, Casein, and Micro Nutrients on Nutritive Value of Soybean for *Tribolium confusum*

Average weight and pupation time of surviving larvae (out of 20) after 15 days at 30° C., 65% relative humidity

Treatment	No. of Larvae	Av. Wt., Mg.	Av. Days to Pupation
Raw soy + 50% starch only	15	0.68	28
Autocl. soy + 50% starch only	19	1.46	22
Raw basal	18	0.82	24
Autocl. basal	18	2.27	18
Raw basal + 0.5% DL-methionine	19	0.60	25
Autocl. basal + 0.5% DL-methionine	19	2.35	18
Raw basal + 10% casein	12	1.07	22
Autocl. basal + 10% casein	19	2.37	18
Whole wheat	17	2.05	19

Recently, Barett and Van Winkle (*3*) reported that electrophoretic and ultracentrifugal analyses of soybean protein indicated that extraction of raw soybean at pH 9.5 resulted in less denaturation than in the acidic milieu usually employed for the purification of soybean inhibitors. The toxicity of such an alkaline extract to *Tribolium* larvae has been determined in addition to the growth-retarding ability of the soybean inhibitors described above.

The multiple nature of the raw soybean-induced growth inhibition observed in vertebrates, and the relative absence of peptic digestion in the larval gut, made it appear likely that *Tribolium* would serve as a fast growing and inexpensive test organism for some of the toxic substances known to occur in soybeans. None of the recognized soybean inhibitors exerted an adverse effect on larval growth. These included the crystalline trypsin inhibitor (*18*), the ethyl-alcohol–soluble inhibitor (*5*), a pH 4.2 extract of soybean (*5*), a kaolin-treated acid extract of soybean (*14*), an alkaline extract of soybean (*3*), and soyin (*22*). It was possible, however, to fractionate soybean to yield a hitherto undescribed material which retarded larval growth to a marked extent.

Procedure

The larvae were reared in 2 × 5 cm. vials as described by Fraenkel and Blewett (*10*). In brief, ten newly hatched larvae were placed on 1 gram of diet and permitted to develop for about 14 days. Each diet was assayed in duplicate. At the end of the incubation period the average weight of the surviving larvae was recorded and the insects were returned to the food. The average length of time necessary for the insects to achieve the pupal stage was subsequently determined by frequent inspection of the diets. Two criteria were available, therefore, for the evaluation of the nutritive value of the rations: the average body weight and the rate at which metamorphosis proceeded.

All the basal diets were fortified with the following growth factors per gram of diet: McCollum-Davis—185 salts mixture 20 mg., cholesterol 10 mg., choline chloride 500 γ, *i*-inositol 250 γ, nicotinic acid 50 γ, thiamine hydrochloride and calcium pantothenate 25 γ, riboflavin and pyridoxine 12.5 γ, folic acid 2.5 γ, and biotin 0.5 γ. The B vitamins were added dissolved in 0.1 ml. of $10^{-4}N$ ammonium hydroxide.

Crystalline soybean trypsin inhibitor was purchased from the Worthington Biochemical Sales Co., Freehold, N. J. The ethyl alcohol–soluble antitrypsin was prepared according to Bowman (*6*). The method of Ham and Sandstedt (*14*) was followed for the preparation of the kaolin-treated antitrypsin. One gram of absorbent was added per 30 grams of raw soybeans extracted. The final purified inhibitor solution was dialyzed and dried by lyophilization. The pH 4.2 extract of raw soybean was prepared by adjusting an 8% suspension of finely ground, untreated soybean meal to pH 4.2 with 40% sulfuric acid. The slurry was stirred for 1 hour at room temperature and centrifuged, and the clear supernatant was dialyzed and lyophilized. The pH 9.5 extract of raw soybean was prepared in the same manner as the acid extract, using 10% sodium hydroxide in place of acid.

Soyin was prepared by the method of Liener and Pallansch (*22*), and showed the following properties:

H_2O, %	14.0
N, % dry wt.	14.7
Toxicity, (LD_{50}, rat), mg./kg.	50
Hemagglutinating titer, γ N	3
Antitryptic activity	Trace

Fractions C and C_1, the soybean inhibitors toxic to *Tribolium* larvae, were prepared as follows: One hundred grams of defatted raw soybean flour were suspended in 900 ml. of water and adjusted to pH 6.7 with a few drops of 10% sodium hydroxide. The suspension was stirred for 45 minutes and centrifuged at 2000 r.p.m. for 10 minutes. The insoluble residue was discarded and the supernatant was brought to pH 4.6 with $10N$ hydrochloric acid. The acid extract was centrifuged and the precipitate discarded. The supernatant was diluted to 700 ml. and 210 grams of ammonium sulfate were added gradually with stirring (0.475 saturation). The insoluble fraction was collected by centrifugation and the supernatant discarded. The precipitate was dissolved in a minimum amount of water, usually 40 ml., and dialyzed in the cold until free of ammonium ion. The entire dialyzed material was designated as fraction C after lyophilization. In one experiment the dialyzed fraction was centrifuged and the lyophilized supernatant was designated as fraction C_1.

The 1-110 trypsin powder used in the feeding trials was obtained from the Pfanstiehl Chemical Co., Waukegan, Ill. The ethyl alcohol–soluble fraction of this powder was prepared by the method of Borchers and Ackerson (*4*). The U.S.P. dried brewer's yeast and the 1-300 trypsin used in the enzymatic studies were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Labco vitamin-free casein was obtained from the Borden Co., New York, N. Y.

The soybean oil meal basal rations contained 50% of hexane-extracted soybean meal (Lincoln var.) grown at the University of Illinois. As used in this paper, the terms "soy," "soybean," or "soybean meal" refer to such material. After ad-

Table II. Effect of Soyin and Crystalline Soybean Trypsin Inhibitor (CSBTI) on Growth of *Tribolium confusum*

Average weight and pupation time of surviving larvae (out of 20) after 14 days at 30° C., 65% relative humidity

	No. of Larvae	Av. Wt., Mg.	Av. Days to Pupation
Raw soy basal	0	..	..
Autoclaved soy basal	20	1.94	18
Autoclaved soy basal + 5.00% raw soy	19	1.51	19
Autoclaved soy basal + 2.00% Kunitz CSBTI	18	1.96	19
Autoclaved soy basal + 2.50% soyin	20	1.54	20
Autoclaved soy basal + 2.00% Kunitz CSBTI + 2.50% soyin	20	1.47	21
Autoclaved soy basal + 5.00% fraction C_1	17	0.59	24

dition of the various experimental materials and the micro nutrients, cornstarch (Coleman and Bell) was added to make 100%. Heat treatment of the soybean meal was carried out by autoclaving the raw meal for 30 minutes at 15 pounds per square inch. The rations containing 19 amino acids were formulated according to Rose *et al.* (*28*) and contained the amino acid mixture 16%, micro nutrients, and starch to make 100%. Additions were made at the expense of the starch.

Proteolytic activity was determined by the azo-casein technique of Charney and Tomarelli (*7*) as used by Powning *et al.* (*27*). The following minor modifications were introduced: Enzyme, inhibitor, and azo-casein solutions were dissolved in 0.1*M* phosphate buffer adjusted to pH 7.4, the incubation time was lengthened to 60 minutes, and undigested protein was removed by centrifugation after addition of the trichloroacetic acid rather than by filtration.

For each determination, standard curves were constructed using appropriate dilutions of 1-300 trypsin and the *Tribolium* enzyme preparation. The relative antitryptic activities of the two enzyme solutions were measured alone and in the presence of inhibitor by reference to the standard curves. The activity of the trypsin solution was adjusted to be equal to or greater than that of the corresponding insect enzyme preparation. Usually a trypsin concentration of 70 to 90 γ per ml. exhibited the same hydrolytic activity as a *Tribolium* extract containing the equivalent of 20 larval digestive tracts per ml.

The larval enzyme preparation was prepared fresh for each experiment. The digestive tracts of late instar larvae were removed by microdissection and placed in a chilled mortar containing 2 ml. of buffer until the full complement of guts had been obtained. The tissues were then ground with a pinch of sand, transferred to a centrifuge tube, and made to volume with buffer. The homogenate was centrifuged for 5 minutes at 1200 r.p.m. and the supernatant was used immediately. The glassware and buffer used in this procedure were kept below 5° at all times. Usually 8 ml. of enzyme solution representing the soluble fraction of 225 digestive tracts were sufficient for one assay, including the standard curve and the requisite blanks.

Results

All of the nutritional experiments described in this paper have been performed with the larger coleopteran, *Tenebrio molitor*, as well as with *Tribolium*. The response of *Tenebrio* larvae was identical to that of *Tribolium*, but the more rapid developmental rate of the latter insect offered a distinct advantage in these feeding trials.

Table I presents the basic phenomena which inaugurated the present study. The poor nutritive value of unsupplemented soybean diets was evident even after fortification of the raw and heated meal with an available carbohydrate, starch. The well known beneficial effect of autoclaving was shown with *Tribolium* by comparison with the natural food of this insect, wheat. It was evident; however, that the deficiencies of soybean in micro nutrients had to be overcome before the full difference between the dietary value of raw and autoclaved soybean could be demonstrated. As the nutritional shortcomings of unsupplemented soybean diets had no direct bearing on the problem of soybean toxicity, and as growth proceeded optimally on the autoclaved basal diet, no effort was made to investigate the specific dietary deficiencies of this legume for *Tribolium*.

Neither 0.5% DL-methionine nor 10% casein stimulated the growth rate significantly. Other experiments in which the raw basal diet was supplemented with those other amino acids, which recent analyses by Kuiken and Lyman (*17*) indicated might be limiting for *Tribolium*, resulted in no improvement in growth. Inclusion of 2% dried liver powder, or enzymatic hydrolyzates of casein or lactalbumin, was also without beneficial effect on larval development.

The results of the feeding trials with the crystalline trypsin inhibitor and with soyin are presented in Table II. The addition of 2% of the crystalline antitrypsin to the diet did not retard larval growth. Data presented by Kunitz (*18*) indicated that this substance was present in raw soybean at about the 1% level. The slight inhibition caused by the feeding of 2.5% soyin did not appear to be significant, since the addition of 5% raw meal checked growth to the same extent as the hemagglutinin. These results indicated that little or no concentration of the factor toxic to *Tribolium* had been effected during the purification of this inhibitor. One of the authors (I. E. L.) has determined that diets including 50% raw soybean meal contain about 1.25% soyin, and tests at this level of hemagglutinin resulted in no reduction whatsoever in the larval growth rate.

Figure 1. Activity of crystalline soybean trypsin inhibitor toward proteolytic enzymes of *Tribolium confusum*

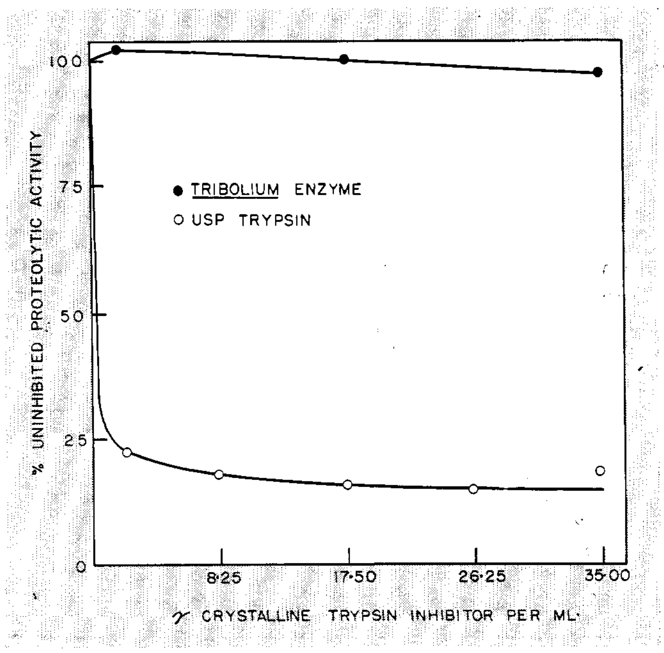

Figure 2. Activity of the ethyl alcohol–soluble trypsin inhibitor toward proteolytic enzymes of *Tribolium confusum*

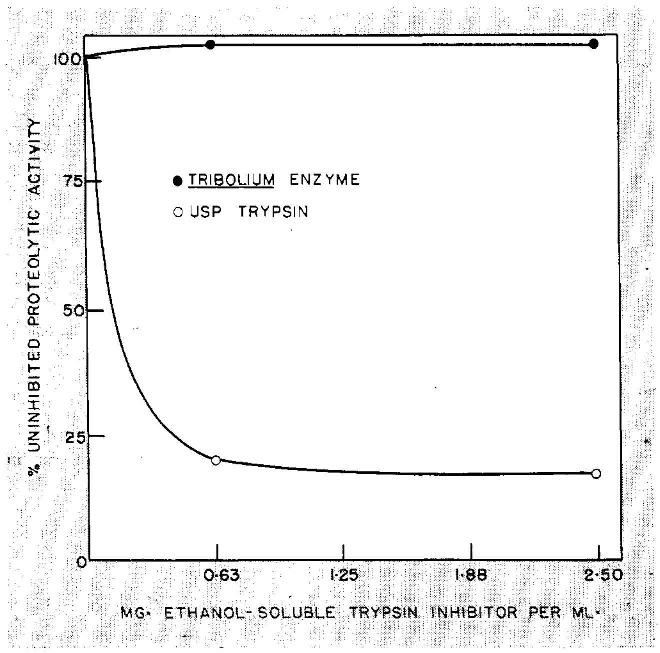

Table III. Effect of Yeast, Trypsin Powder, and Amino Acid Mixtures on Growth of *Tribolium confusum* Receiving Raw Soybean in Diet

Average weight and pupation time of surviving larvae (out of 20) after 13 days at 34° C., 70% relative humidity

	No. of Larvae	Av. Wt., Mg.	Av. Days to Pupation
Raw soy basal	0	. .	. .
+3% brewer's yeast	17	1.79	20
+5% 1–110 trypsin powder	0	. .	. .
+5% ethyl alcohol fraction of trypsin powder	2	1.50	67
Autoclaved soy basal	16	2.20	17
+5% pH 4.2 kaolin-treated trypsin inhibitor	19	2.22	17
Rose's 19 amino acids basal + 5% raw soy	13	0.85	27
+5% autoclaved soy	16	2.09	20

The data in Table II also showed that diets containing both soyin and Kunitz's antitrypsin in levels greater than those occurring naturally in soybean were satisfactory for *Tribolium*. The toxicity of a hitherto undescribed soybean inhibitor was also demonstrated in this experiment. Growth rates were severely curtailed in the presence of 5% of fraction C_1, the material precipitated by adjusting the pH 4.6 extract of raw soybean to 0.475 saturation with ammonium sulfate. Very fresh preparations of the closely related fraction C have subsequently been shown to be lethal to all the larvae receiving this material in the diet at the 2.5% level. Additional study demonstrated that this toxin was inactivated by autoclaving at 15-pound pressure at 121° C.

Further experiments with known soybean inhibitors and materials that antagonized these substances are presented in Table III. The kaolin-treated trypsin inhibitor described by Ham *et al.* (*14*) did not exert a deleterious effect on larval growth, although Powning *et al* (*27*) showed that this antitrypsin could inhibit the proteolytic enzymes of the closely related insect, *Tenebrio molitor*. This failure of antitrypsins to retard growth was corroborated by the studies in which crude trypsin powder, or the ethyl alcohol–soluble fraction of this powder described by Borchers and Ackerson (*4*), were shown to be ineffective in reversing the raw soybean effect in *Tribolium*. An indication that a toxic substance was involved whose function was not primarily the inhibition of digestive enzymes was obtained from the results with the amino acid diets and also from the demonstration that the addition of 3% yeast to the raw diet counteracted most of the toxic effect of raw soybean.

Table IV showed the ineffectiveness of the alcohol-soluble antitrypsin described by Bowman (*6*) as an inhibitor of larval growth. A pH 4.2 extract not treated with kaolin was similarly devoid of toxicity, as was the alkaline extract described by Barett and Van Winkle (*3*). In view of the origin of fraction C_1, it is likely that the acid extract could have been shown to exert a toxic effect if fed at higher levels.

The results of the dietary studies indicated that the failure of *Tribolium* to grow on raw soybean was not due to the action of known soybean inhibitors, but rather to an uncharacterized toxin or toxins present in the raw meal. Much the same results were obtained in the enzymatic studies. Figures 1 and 2 showed the insensitivity of the proteolytic enzymes of *Tribolium* to the action of both the crystalline and the ethyl alcohol–soluble inhibitor. The 1–300 trypsin showed a marked reduction in activity in the presence of identical concentrations of these substances, however. Similar results were obtained with the kaolin-treated acid extract (*14*), which has been shown to inhibit the digestion of protein in *Tenebrio* larvae (*27*).

It can be seen from Figures 3 and 4 that soybean meal contained an inhibitor of proteolysis in *Tribolium* distinct from those previously shown to reduce the activity of vertebrate trypsin. Within the limits of the method used, both the insect and vertebrate enzyme preparations showed about the same degree of inhibition in the presence of both fraction C_1 and the pH 4.2 extract. The relative concentrations of these two fractions required to induce a 50% inhibition, about 7 and 20 γ per ml., respectively, indicated that the antiproteolytic activity of the pH 4.2 extract was probably due in part to the presence of fraction C_1, the ammonium sulfate precipitate of a similar acid extract of raw soybean, in the pH 4.2–soluble material.

Discussion

The response of *Tribolium* larvae was noticeably different from that of vertebrates in several respects. In view of the reliance of insect pests of stored grains on proteolytic enzymes with neutral pH optima, the refractory nature of these enzymes to inhibition by the recognized soybean antitrypsins was unexpected. The lack of toxicity on the part of soyin, on the other hand, might have been predicted on the basis of the known physiology of *Tribolium*. The blood of insects does not contain the erythrocyte type of cell, and the larvae rely entirely upon the tracheal type of oxygen transport system.

Although methionine has been shown to be required for the development of *Tribolium* larvae (*13*, *19*), the data show that the growth rate of this organism was not limited by the unavailability of this nutrient from raw soybean. This marked another distinct deviation from results obtained with rats and chicks (*21*, *25*), which demonstrated that methionine supplements to raw soybean diets improved the growth rate in spite of the influence of inhibitors present in raw soybean to limit the nutritive value of the raw legume.

Borchers and Ackerson (*4*) reported that the addition of 5% yeast to diets containing 25% raw meal was without beneficial effect on rat growth. It would appear that the favorable effect of yeast in the case of *Tribolium* was due to an antagonism of the principle in raw soybean which was toxic to the larvae. Additional study of this effect revealed that neither the water-soluble (100°) nor the water-insoluble portion of dried yeast could replace the intact microorganism. These fractions when recombined were similarly devoid of any growth-stimulating effect, indicating that the therapeutic factor which was present in the yeast may have failed to withstand the extraction treatment.

There is a general but unrecorded impression among insect nutritionists that

Table IV. Effect of Crude Soybean Inhibitors on Growth of *Tribolium confusum*

Average weight and pupation time of surviving larvae (out of 20) after 17 days at 33° C., 70% relative humidity

	No. of Larvae	Av. Wt., Mg.	Av. Days to Pupation
Raw soy basal	2	1.25	25
Autocl. soy basal	15	2.20	19
+5% raw soy	11	1.81	22
+5% ethyl alcohol–soluble trypsin inhibitor	17	2.17	21
+5% pH 4.2 extract, dialyzed and lyophilized	16	2.20	18
+5% pH 9.5 extract, dialyzed and lyophilized	12	2.16	20

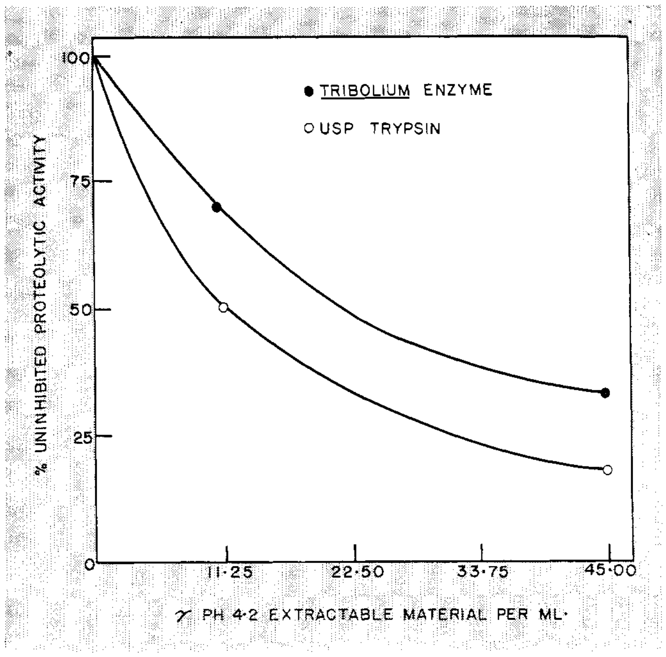

Figure 3. Activity of pH 4.2–soluble fraction of raw soybeans toward proteolytic enzymes of *Tribolium confusum*

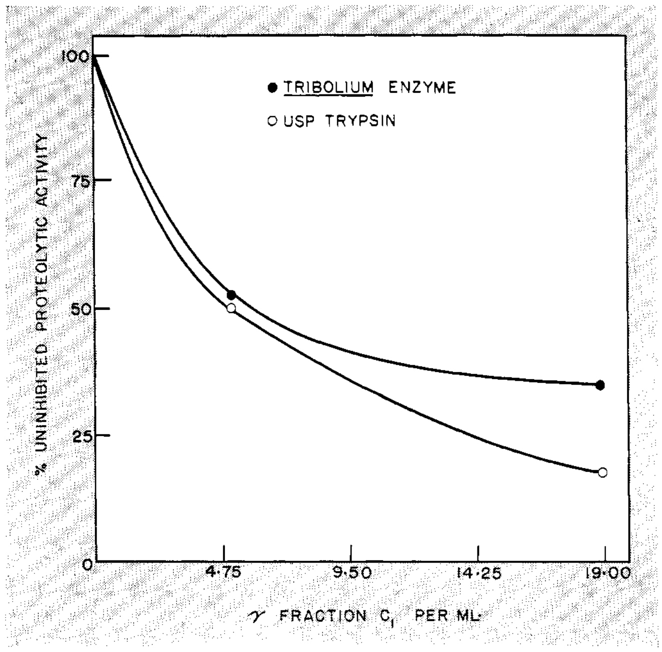

Figure 4. Activity of fraction C_I toward proteolytic enzymes of *Tribolium confusum*

pests of stored products possess specific sensory mechanisms which enable them to select intact yeast particles from the diet at the expense of the remainder of the ration. If such a phenomenon had been operative in the present instance the larvae would have ingested more yeast and less raw soybean than the control animals, and the failure of the crude yeast fractions to stimulate growth lent some support to this concept.

The results of the experiments in which it was shown that raw soybean was able to induce a growth inhibition when included in diets where the sources of nitrogen were crystalline amino acids indicated that the antiproteolytic ability of fraction C did not account for all the growth-inhibiting properties of raw soybean. These findings extend to insects the results obtained in similar studies by Desikachar and De (*9*), Westfall *et al.* (*29*), and Hill and associates (*16*). In the absence of evidence to the contrary, the studies of Liener and others (*2*, *20*) showed that part, if not the majority, of the trypsin-insensitive toxicity of raw soybean to vertebrates was due to the presence of soyin in the raw flour or crude acid extracts of raw soybean administered by the three groups of workers mentioned above. The role of additional growth inhibitors present in raw soybean cannot be discounted, however, and it is possible that the sensitivity of *Tribolium*, *Tenebrio*, and other insects to these hitherto undescribed toxins contribute to the relative freedom of soybean and soybean products from insect damage. The importance of soybean inhibitors which are toxic to insects in the growth retardations observed in higher animals will be the subject of future investigations.

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