Insecticidal Action of the Phytohemagglutinin in Black Beans on a Bruchid Beetle

Abstract. *Black bean (*Phaseolus vulgaris*) phytohemagglutinin added to the normal diet of a bruchid beetle (*Callosobruchus maculatus*) that can eat phytohemagglutinin-free cowpeas (*Vigna unguiculata*) but not* P. vulgaris *seeds kills the bruchid larvae. Trypsin inhibitors, found in both* P. vulgaris *and* V. unguiculata *seeds, have virtually no effect on the bruchid larvae. The conclusion is that a major part of the adaptive significance of phytohemagglutinins in black bean and other legume seeds is to protect them from attack by insect seed predators.*

Mature black bean seeds (*Phaseolus vulgaris* L.) are a staple protein source for man in much of the neotropics. They must be cooked before eating. It appears that this heating inactivates the two proteinaceous toxic compounds in black beans, a trypsin inhibitor (*1*) and a phytohemagglutinin (*2, 3*). This report deals with the adaptive significance of the phytohemagglutinin in black bean seeds.

Many untreated phytohemagglutinins (lectins) (*2, 4*) are toxic when administered intravenously or orally to domestic mammals and birds (*2, 3*). Therefore it may be argued that phytohemagglutinins are adaptive in reducing predation on wild legume seeds by wild vertebrates. While probable, this hypothesis has never been tested. However, several hundred species of neotropical legume seeds are eaten by the larvae of bruchid beetles (*5, 6*). Wild *Phaseolus* are no exception, and at least ten species of bruchids are suspected or known to feed on *P. vulgaris* seeds in various parts of the world (*6*). It would therefore appear that the phytohemagglutinins in black bean seeds are of no adaptive significance in reducing predation by bruchids. However, neotropical bruchids are extremely species-specific, with the great majority of them having only one species of prey in a given habitat (*5*). The bruchids' extreme prey-specificity reduces seed predation on any given prey species because nearly all of the species of bruchids in a given habitat cannot feed on any given species of potential prey. For example, when grown in lowland Costa Rican habitats containing at least 75 species of wild bruchids, *P. vulgaris* seeds are not eaten by any bruchids in the field (*7*).

We postulate that the phytohemagglutinin in black beans is of adaptive significance in being one of the major reasons why these bruchids do not prey on *P. vulgaris* seeds (*8*). The ideal test of this hypothesis would be to demonstrate that the larvae of most bruchids in the habitat of *P. vulgaris* are killed by the inclusion of black bean phytohemagglutinin in their diet. Such a test is technologically impossible at present because none of the 75 or more relevant species of wild bruchids are maintained in persistent laboratory culture (*9*). In view of this technological impasse, the next best step is to show that black bean phytohemagglutinin is toxic to at least one bruchid. *Callosobruchus maculatus* (Fabricius), the southern cowpea weevil (*10*), is easily reared in the laboratory (*11*), and its larvae die when they attempt to feed on black beans (*12*). This mortality could be due to the phytohemagglutinin, the trypsin inhibitor, or to the other toxic secondary compounds found in *P. vulgaris* seeds (*8*). The following experiment was designed to determine if either of the former compounds is toxic to *C. maculatus* larvae. We suspected the phytohemagglutinin would be toxic because cowpeas do not show phytohemagglutinin activity (*13*) and therefore the bruchid is not likely to have evolved detoxification mechanisms.

Artificial seeds were made of cowpea flour (*14*). In the experimental seeds, dry, powdered black bean phytohemagglutinin (*15*) was added at concentrations of 0.1, 1, and 5 percent. These concentrations were chosen because legume seeds generally contain 0.6 to 2.0 percent (dry weight) phytohemagglutinin if they contain it at all (*16*). The female *C. maculatus* oviposited readily on the control and experimental artificial seeds; all but ten eggs were removed from each artificial seed, and the seeds were incubated for 73 to 80 days to ensure the emergence of all surviving beetles. Control artificial seeds produced an average of 4.5 beetles per seed (N = 14; S.D. = 1.7). Experimental artificial seeds with 0.1 percent phytohemagglutinin produced 3.6 beetles per seed (N = 14; S.D. = 0.4), which is a significantly smaller number of beetles than from control seeds (t = 1.931; 26 d.f., one-tailed test). Two dwarfed adults were produced from 14 artificial seeds containing 1 percent phytohemagglutinin. No beetles survived to adulthood in 14 artificial seeds with 5 percent black bean phytohemagglutinin. The experiment was repeated with five artificial seeds in each category. The control seeds produced a total of 4.6 beetles per seed; the 0.1 percent phytohemagglutinin diet produced 3.2 beetles per seed; the 1 percent phytohemagglutinin diet produced 0.4 beetle per seed and the 5 percent phytohemagglutinin diet produced no beetles. To determine whether it was the simple addition of a protein to the diet that was having the negative effect on beetle larvae, the same test was made with heat-inactivated black bean phytohemagglutinin (*17*). Four artificial seeds were used in each of five categories: control, 0.1, 1, 5, and 10 percent black bean phytohemagglutinin in the artificial seeds. They produced 6.0, 6.5, 5.8, 4.5, and 4.5 beetles per seed. This dramatic increase over the results obtained with untreated black bean phytohemagglutinin shows that it is not merely the addition of a novel protein to the diet that reduced the number of survivors.

It seemed unlikely that the trypsin inhibitor in black beans would be as toxic to *C. maculatus* larvae as the phytohemagglutinin in that cowpeas contain trypsin and chymotrypsin inhibitors (*18*). Furthermore, Applebaum has convincingly argued (*19*) that bruchids probably represent an adaptive radiation onto legume seeds that are rich in trypsin (protease) inhibitors since they lack proteases in their gut contents. However, to eliminate the possibility that black beans are toxic to bruchids by virtue of their trypsin inhibitors, we added commercially available trypsin inhibitors to the artificial seeds. Egg white trypsin inhibitor or soybean trypsin inhibitor (*20*) at concentrations of 0.1 and 1 percent did not affect the beetles; at 5 percent concentration, egg white trypsin inhibitor had no effect, while soybean trypsin inhibitor reduced beetle production by only 21 percent.

Hypotheses regarding the role of phytohemagglutinins in plants have been many and varied (*4, 16*) and include the following: (i) protect the plants against fungal attack, (ii) transport sugar, (iii) attach glycoprotein enzymes in organized multienzyme systems, and (iv) mediate the symbiotic relationship between legumes and soil bacteria. All except (i) are unlikely to be operative in a dry and quiescent legume seed. To this list we can add the observation that at least one phytohemagglutinin is toxic to an insect that regularly feeds on legume seeds. Legume seeds contain a variety of kinds and concentrations of phytohemagglutinins, and a major cause of mortality of wild legume seeds is predation by highly host-specific bruchid beetles. We conclude that a major part of the adaptive significance of phytohemagglutinin to *P.*

vulgaris and other legume seeds is to protect them from attack by insect seed-predators. Such a conclusion is of conspicuous significance to agriculturalists. Selection for those strains of black beans free of phytohemagglutinins (*21*) so as to reduce bean processing costs would very likely produce a crop plant on which many wild bruchids could then feed.

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References

1. I. E. Liener and M. L. Kakade, in *Toxic Constituents of Plant Foodstuffs*, I. E. Liener, Ed. (Academic Press, New York, 1969); A. C. Leopold and R. Ardrey, *Science* **176**, 512 (1972).
2. I. E. Liener, *Agr. Food Chem.* **22**, 17 (1974).
3. W. G. Jaffé, in *Toxicants Occurring Naturally in Foods* (National Academy of Sciences, Washington, D.C., 1973).
4. N. Sharon and H. Lis, *Science* **177**, 949 (1972); H. Lis and N. Sharon, *Annu. Rev. Biochem.* **42**, 541 (1973).
5. D. H. Janzen, In *Evolutionary Strategies of Parasitic Insects and Mites*, P. W. Price, Ed. (Plenum, New York, 1975), p. 154.
6. F. Zacher, *Z. Angew. Entomol.* **33**, 460 (1952).
7. Unpublished records in a general study of Costa Rican bruchids and their host plants.
8. There are other defensive compounds in *P. vulgaris* as well, but they are not the subject of this report. Heteropolysaccharides and saponins that are toxic to bruchids occur in *P. vulgaris* seeds [S. W. Applebaum, U. Tadmor, H. Podoler, *Entomol. Exp. Appl.* **13**, 61 (1970)].
9. However, wild bruchids (*Mimosestes sallaei*, *M. immunis*, and *Stator limbatus*) from Guanacaste Province, Costa Rica, have oviposited on mature and shelled black beans in the laboratory, and the larvae always died very shortly after mining into the seeds.
10. Misnamed a "weevil" long ago, this animal is in fact a member of the family Bruchidae, a family long known as "seed weevils."
11. Stock cultures are maintained in quart mason jars full of mature commercial cowpeas, *Vigna unguiculata* (L.) (a synonym of *Vigna sinensis*).
12. If seeds of black beans or other cultivars of *P. vulgaris* are mixed with the stock cowpeas, eggs are laid on them but all larvae die shortly after mining into the *P. vulgaris* beans.
13. Determined in the laboratory of I.E. Liener.
14. See (*5*) for detailed methods.
15. Prepared according to P. M. Honovar, C.-V. Shih, I. E. Liener, *J. Nutr.* **77**, 109 (1962).
16. I. E. Liener, *Annu. Rev. Plant Physiol.*, in press.
17. Hemagglutinating activity was destroyed by autoclaving a concentrated solution of the phytohemagglutinin at 15 pounds pressure (1 atm) at 130°C for 20 minutes followed by lyophilization.
18. M. M. Ventura and J. X. Filho, *An. Acad. Bras. Cienc.* **38**, 553 (1966).
19. S. W. Applebaum, *J. Insect Physiol.* **10**, 783 (1964).
20. Trypsin inhibitors (soybean and ovomucoid) were purchased from Sigma Chemical Company, St. Louis, Mo.
21. O. Brucher, M. Wecksler, A. Levy, A. Palozzo, W. G. Jaffé, *Phytochemistry* **8**, 1739 (1969); W. G. Jaffé, O. Brucher, A. Palozzo, *Z. Immununitaetsforsch. Allerg. Klin. Immunol.* **142**, 439 (1972).
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DNA Structure in Sheared and Unsheared Chromatin

Abstract. *Shearing chromatin, by either sonication or vortex homogenization, introduces significant structural artifacts. These may be detected by the anomalously large increase in the number of ethidium bromide binding sites and the large alteration of the circular dichroism spectra of chromatin. Structural alterations are also suggested by the disappearance of differential light scattering after shearing.*

The term chromatin in this report refers to the isolated chromosomes (*1*) and has only an operational definition since chromatin preparations vary from one laboratory to another. Chromatin studies would be facilitated if the material could be obtained in a homogeneous solution, and for this reason most workers have studied the functional and structural properties of chromatin after shearing it, either with a motor-driven homogenizer (*2*) or by sonication (*3*). However, it has been reported that shearing causes a dramatic change in template activity (*4*) and loss of the repeating units structure of chromatin (*5*). Unfortunately, unsheared chromatin forms a suspension and gives rise to large light-scattering artifacts. Since our previous data on chromatin changes in proliferating cells (*6*) were obtained with unsheared chromatin, we have developed and present here a method to correct for light-scattering artifacts (*7*). With this method we have compared sheared and unsheared chromatins from various cell types, using circular dichroism (CD) spectra (*6, 8*) and ethidium bromide binding (*6, 9–11*) as indications of chromatin structure.

The following cells were used: (i) WI-38 human diploid fibroblasts in confluent monolayers, either resting or stimulated to proliferate (*10*); (ii) AF-8 cells, a temperature-sensitive mutant of BHK cells (*12*), which grow at 34°C but are arrested in G_1 at 39°C; and (iii) HeLa S-3 cells maintained in suspension and synchronized by selective detachment (*13*). Chromatin was prepared as previously described (*10, 13*). Unsheared chromatin was prepared by gently resuspending the viscous chromatin pellet, with or without a few strokes of a Dounce homogenizer; sheared chromatin was prepared by resuspending the pellet and either sonicating at 50 watts for 20 seconds or more or homogenizing with a motor-driven homogenizer for 15 seconds or more. In all three cases, the solvent was 0.01*M* tris(hydroxymethyl)aminomethane hydrochloride (tris-HCl), *p*H 8.0. Circular dichroism spectra were obtained with a Jasco model J-40 recording spectropolarimeter as previously described (*10, 11, 13*). The mean ellipticity, θ, is expressed as degrees times centimeters squared per decimole of nucleotide residue, assuming a mean molecular weight of 330. The ellipticity obtained directly from the recorder chart is designated as ψ.

The CD instrument is very sensitive to scattering artifacts. Because of its particulate nature, chromatin tends to form an intensely light-scattering suspension.

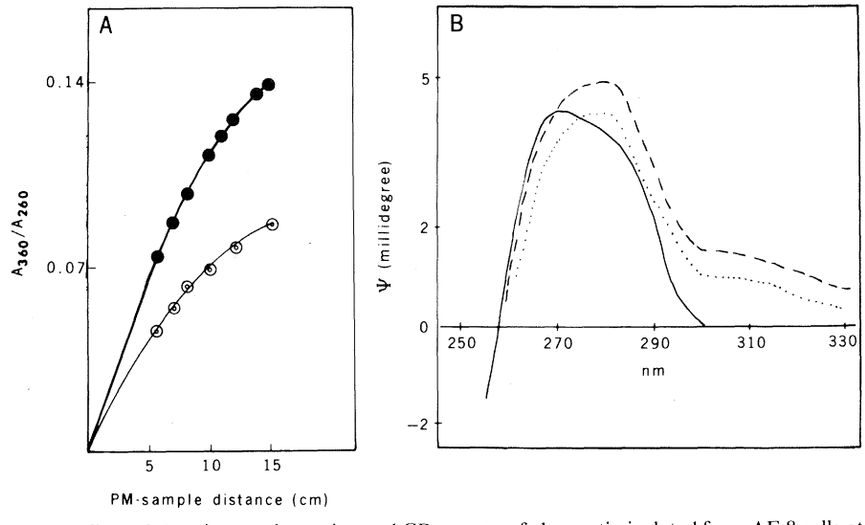

Fig. 1. Effect of shearing on absorption and CD spectra of chromatin isolated from AF-8 cells at 39°C. (A) Absorbance ratio as a function of PM-sample distance for unsheared (closed circles) and sheared (open circles) chromatins. The unsheared chromatin was subjected to a few strokes of a Dounce homogenizer and the sheared chromatin was sonicated for 20 seconds at 50 watts. (B) Observed CD spectra of unsheared chromatin at D = 14 cm (dashed line) and 7 cm (dotted line) and of sheared chromatin at D = 14 cm (solid line). Standardization and calibration are described elsewhere (*7, 17*).