

Toxicity of Purothionin and Its Homologues to the Tobacco Hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae)¹

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Toxicity of Purothionin and its Homologues to the Tobacco Hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). KRAMER, K. J., KLASSEN, L. W., JONES, B. L., SPEIRS, R. D., AND KAMMER, A. E. (1979). *Toxicol. Appl. Pharmacol.* 48, 179-183. Purothionin from wheat or homologues from barley and rye were injected into the hemocoel of larvae of *Manduca sexta* and caused 50% mortality at doses of 17-46 µg/g. Applications at 2-50 µg purothionin to a *Manduca* moth nerve-flight muscle preparation produced a rapid, dose-dependent depolarization of the muscle-fiber membrane. The α-isopeptides were two- to threefold more toxic than the β-forms. Alkylation of the cysteine residues of the peptides abolished both activities.

Wheat, barley, and rye seeds contain basic polypeptides of relatively low molecular weight, called purothionin and purothionin homologues, that are toxic to vertebrates and to some bacteria and yeasts (Coulson *et al.*, 1942; Stuart and Harris, 1942; Fernandez de Caley *et al.*, 1972; Hernandez-Lucas *et al.*, 1974, 1978; Ohtani *et al.*, 1975). Multiple forms of these peptides (α and β) have been isolated that differ in only a few amino acid residues (Mak and Jones, 1976a; Jones and Mak, 1977). No data are as yet available concerning the toxicity of these materials toward invertebrates. Although much is

known about the physical and chemical properties of these peptides (Redman and Fisher, 1968; Okada *et al.*, 1970; Okada and Yoshizumi, 1973; Nimmo *et al.*, 1974; Mak and Jones, 1976a,b; Jones and Mak, 1977), there is little information concerning the mechanism of action in animals or the *in vivo* function in seeds. In our laboratory we are examining both the insecticidal properties and the biochemical mode of action of these peptides. As a first step we have developed two sensitive bioassays using the tobacco hornworm, *Manduca sexta* (L.), that will be used to investigate the toxicity and pharmacological action of the polypeptides. The first bioassay is used to determine lethality when the peptides are injected into larvae; the second is used to measure electrophysiologically their effects on membrane depolarization of the dorsal longitudinal muscle from the adult moth.

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METHODS

Purothionin, α and β , was extracted and purified from wheat flour by the method described previously by Mak and Jones (1976a,b). The flours were milled from *Triticum aestivum* L. (cv Manitou), *T. durum* Desf. (cv Steward 63), and *T. monococcum* L. The barley homologue, hordothionin, was similarly extracted from flour milled from barley (*Hordeum vulgare* L. cv Conquest). The rye homologue of purothionin was extracted from rye (*Secale cereale* L.) flour with 0.05 M H_2SO_4 and precipitated with 12% (w/v) trichloroacetic acid. The precipitated protein was dissolved in water, extracted with diethyl ether to remove contaminating lipids, adjusted to pH 5.2 with NH_4OH , clarified by centrifugation, and separated from contaminating proteins by elution from carboxymethyl (CM) cellulose following the procedure used for wheat purothionins. The *S*-pyridylethylated derivative of purothionin from *T. aestivum* was prepared by using 4-vinyl pyridine (Mak and Jones, 1976b).

Peptide concentration was measured by absorbance at 278 nm by using $\epsilon_{278} = 1300 M^{-1} cm^{-1}$ calculated on the basis of 1 mol each of tyrosine and phenylalanine/mol peptide (Mihalyi, 1970; Nimmo et al., 1974; Mak and Jones, 1976a,b; Ohtani et al., 1975; Jones and Mak, 1977). The concentration of *S*-pyridylethylated purothionin was estimated by dry weight.

M. sexta eggs were obtained from Dr. J. P. Reinecke (Metabolism and Radiation Research Laboratory, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Fargo, N. Dak.). Larvae were reared at 28°C and 60% relative humidity with a 16-hr light photoperiod and a standard diet (Bell and Joachim, 1976). The confused flour beetle, *Tribolium confusum* Jacquelin duVal, was obtained from a laboratory culture maintained at the U.S. Grain Marketing Research Laboratory.

Tests of lethality were performed on larvae that were in the final day of the fourth instar (0.9 ± 0.2 g) by injecting the sample (1–500 $\mu g/50 \mu l$ per animal) dissolved in insect saline solution (128 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$) through a proleg of the sixth abdominal segment. The test animals were observed at 72 hr and LD50 values determined using probit analysis according to Finney (1952). Decreasing doses of toxic material were administered to groups of 2–10 test animals depending on the supply of peptide available for testing. Growth rates were determined by measuring the mean weight of treated or control animals at 1 to 4-day intervals for about 20 days postinjection.

The effect of oral administration of the peptide was examined by blending 50–500 ppm of the test material thoroughly into freshly prepared *M. sexta* diet (Bell

and Joachim 1976) cooled to 50°C. Then each neonate larva was given approximately 13 g of treated or untreated diet. The mean weight of 10 animals fed control or toxin-supplemented diet was measured at 1- to 4-day intervals for about 40 days. The flour beetles were fed toxin admixed with enriched wheat flour at similar concentrations.

Moths that were 1 day post-pupal–adult ecdysis were used for studying the effects of the polypeptides on nerve–muscle preparations. Immobilization of the moths for surgery was attained by taping them ventral side up to a waxed dissecting dish. The muscle fibers were exposed by cutting along the ventral midline of the thorax and then gently pulling the cuticle and underlying muscle aside with small hooks made from insect pins. The Malpighian tubules which lie ventrally to the dorsal longitudinal muscle were removed with a forceps and a small hole was made in the trachea overlying the muscle to allow penetration of the fibers with the electrode. During the dissection the exposed tissue was bathed in physiological saline (pH 7.0) composed of 25 mM NaCl, 25 mM potassium methane sulfonate, 4 mM $CaCl_2$, 33 mM $MgCl_2$, 150 mM Trismethane sulfonate (modified after Rheuben, 1972). Preparations exposed to saline remained viable for several hours after the dissection.

Intracellular potentials were recorded by oscilloscope (Tectronics, Model 5103N, and W. P. Instruments Co., Model M4A high impedance amplifier) from the superficial muscle fibers by means of micropipet electrodes containing 3 M KCl. Fibers that did not maintain a membrane potential of at least –50 mV for 5 min at the beginning of the recording period were not used. Purothionin was applied by replacing the saline solution used during dissection with 0.1 ml of the appropriate concentration of peptide dissolved in insect saline solution. Because it was not possible to remove all the dissecting solution completely, the concentration of purothionin applied to the muscle is only an estimate. The toxicities of the α - and β -isopeptides were compared by observing the change in membrane potential of one superficial fiber as a function of peptide concentration over a 5-min period. After a decrease in potential was observed, many other superficial fibers were penetrated to see if the depolarization was uniform. Control animals maintained potentials of 50–60 mV for at least 30 min and usually longer than 1 hr.

RESULTS

When as little as 5 $\mu g/g$ purothionin- α or - β was injected into fourth-instar larvae of *M. sexta*, many larvae became disabled within a few hours. The observed results

depended on the amount of toxin injected. Sublethal quantities led to phlegmatic behavior, that is, the test animals lay motionless on their sides. A characteristic lethal sign observable within a few hours postinjection was a blackening (melanization) of the insect cuticle.

The peptides from the three wheat cultivars were all moderately toxic to *M. sexta* when injected into the hemocoel; LD50 values ranged from 19 to 46 $\mu\text{g/g}$ (Table 1). The toxicity fell into two general classes. The β -form (less basic) of the peptide was approximately half as toxic as the α -peptide (Mak and Jones, 1976b). The homologous peptides from barley and rye displayed similar toxicities. These values were about 10-fold higher than the effective dose for the pig (LD50 = 1.6 mg/kg); rabbits are about 2-fold more susceptible than the hornworm (Coulson *et al.*, 1942).

We also administered as much as 500 ppm of the wheat peptides orally (in the diet) to *M. sexta* and *T. confusum*. However, this treatment had no effect on growth or development of either species. Peptidases in the insect digestive tract apparently degraded purothionin to smaller nontoxic products as is the case with most peptide materials. The barley and rye homologues were not tested for oral toxicity.

TABLE 1

THE LETHAL POTENCY OF INJECTED PUROTHIONIN AND ITS HOMOLOGOUS POLYPEPTIDES TO *M. sexta* LARVAE^a

Peptide	LD50 ^b ($\mu\text{g/g}$)
<i>T. aestivum</i> , α	19 (15-28)
<i>T. aestivum</i> , β	36 (28-51)
<i>T. durum</i> , α	23 (19-35)
<i>T. durum</i> , β	46 (34-78)
<i>T. monococcum</i> , β	~44
<i>H. vulgare</i> , α	~17
<i>H. vulgare</i> , β	~45
<i>S. cereale</i>	~42
<i>T. aestivum</i> α -S-pyridylethylated	> 520

^a Fourth-instar larvae weighing 1.0 ± 0.2 g.

^b The 95% confidence limits given in parentheses.

TABLE 2

THE EFFECT OF *T. aestivum* PUROTHIONIN ON *Manduca* FLIGHT MUSCLE MEMBRANE POTENTIAL^a

Peptide	Amount applied (μg) ^b	Number of Animals	Average positive change in membrane potential (mV) ^b
Saline control	—	5	0
α	50	1	55
	25	1	55
	10	1	55
	5.0	5	44 \pm 9
	3.5	3	17 \pm 6
	2.5	2	0
β	25	2	55
	20	1	55
	17	1	50
	15	1	55
	14	3	48 \pm 4
	12	4	10 \pm 10
	10	3	7 \pm 9
α -S-Pyridylethylated	500	2	0

^a Normal potential ranged from -50 to -60 mV.

^b Five-minute observation period. Mean \pm SD.

When purothionin- α or - β was added to the bathing solution of an adult flight muscle fiber (2.5-500 μg), the resting potentials of the fiber decreased in a dose-dependent fashion (Table 2). Relatively small amounts of either peptide (3-15 μg) disrupted the membrane potential, but the α -peptide was approximately two- to threefold more effective than the β -form. The depolarization effect was irreversible; continuous washing of the tissue with purothionin-free saline solution for 30 min did not restore the resting potential. These data were symbatic with the acute toxicity results obtained with treated larvae (Table 1). The results suggest that the mode of action of these toxic peptides involves at least in part an effect on the permeability of cell membranes.

DISCUSSION

Now that two bioassays for purothionin and its homologues are available, a more

detailed investigation into the mechanism of action of the peptides will be facilitated. Our experimental animal is an insect. We utilize the hornworm because it is readily available, easy to handle, and quite small, the latter allowing us to assay very small doses of material. Not only are these peptides toxic to insects, but they also possess toxicity to vertebrates and microorganisms (Coulson *et al.*, 1942; Stuart and Harris, 1942; Fernandez de Caleyá *et al.*, 1972; Hernandez-Lucas *et al.*, 1974; Ohtani *et al.*, 1975). We believe that the toxicological and electrophysiological results obtained using our invertebrate animal are relevant to an understanding of the effects of these peptides in all organisms. Using the yeast *Saccharomyces carlsbergensis*, Okada and Yoshizami (1973) demonstrated that both the wheat and barley peptides adsorb to the cell membrane and disrupt carbohydrate, protein, nucleotide, and ion transport. The electrophysiological evidence reported here supports this hypothesis that the peptides interact with the cell membrane and produce a change in permeability.

The unique toxicities of the α - and β -isopeptides are no doubt caused by the 10% difference in amino acid sequence (5 out of 45 residues, Mak and Jones, 1976b) and indicate that primary modifications in structure result in activity changes. We do not know which amino acid residues are most critical for functionality. Preliminary studies have determined that the cystine residues of purothionin and/or the native conformation of the peptide are essential for both toxic and membrane activities; when these residues were alkylated with 4-vinyl pyridine, the modified material was not functional in either the larval or adult assay, even at a concentration 50-fold the minimum effective dose of the native toxin (Tables 1 and 2). However, the pyridylethylated derivative did have a separate effect on the moth, since it caused continuous contraction of the flight muscles. In dissected preparations such contraction was abolished by cutting the

motor nerves to the muscle, which suggested that the modified peptide acted on the nervous system rather than directly on the muscles. In an intact moth, injection of 500 μ g of the S-pyridylethylated derivative also caused continuous flight for 15 min, followed by quiescent behavior like that observed after less than 30 sec in saline-injected controls. Thus, the peptide has two distinct physiological effects, depending on whether or not it was chemically modified. The bioassays described in this report will be used to explore further the toxicity and pharmacological action of additional chemical derivatives of the polypeptides in the hope of learning more about their mode of action.

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REFERENCES

- BELL, R. A., AND JOACHIM, F. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Amer.* **69**, 365-373.
- COULSON, E. J., HARRIS, T. H., AND AXELROD, B. (1942). Effect on small laboratory animals of the injection of the crystalline hydrochloride of a sulfur protein from wheat flour. *Cereal Chem.* **19**, 301-308.
- FERNANDEZ DE CALEYA, R., GONZALEZ-PASCUAL, B., GARCIA-OLMEDO, F., AND CARBONERO, P. (1972). Susceptibility of phytopathogenic bacteria to wheat purothionin *in vitro*. *Appl. Microbiol.* **23**, 998-1000.
- FINNEY, D. J. (1952). *Probit Analysis*, 2nd ed. Cambridge Univ. Press, London.
- HERNANDEZ-LUCAS, C., CARBONERO, P., AND GARCIA-OLMEDO, F. (1978). Identification and purification of a purothionin homologue from rye (*Secale cereale* L.). *J. Agr. Food Chem.* **26**, 794-796.
- HERNANDEZ-LUCAS, C., FERNANDEZ DE CALEYA, R., AND CARBONERO, P. (1974). Inhibition of brewer's yeasts by wheat purothionins. *Appl. Microbiol.* **28**, 165-168.

- JONES, B. L., AND MAK, A. S. (1977). Amino acid sequences of the two α -purothionins of hexaploid wheat. *Cereal Chem.* **54**, 511-523.
- MAK, A. S., AND JONES, B. L. (1976) a. Separation and characterization of chymotryptic peptides from α - and β -purothionins of wheat. *J. Sci. Food Agr.* **27**, 205-213.
- MAK, A. S., AND JONES, B. L. (1976) b. The amino acid sequence of wheat β -purothionin. *Canad. J. Biochem.* **54**, 835-842.
- MIHALYI, E. (1970). Numerical values of the absorbancies of the aromatic amino acids in acid, neutral and alkaline solution. In *Handbook of Biochemistry* (H. A. Sober, ed.), pp. B75-76. The Chemical Rubber Co., Cleveland, Ohio.
- NIMMO, C. C., KASARDA, D. D., AND LEW, J. L. (1974). Physical characterization of the wheat protein purothionin. *J. Sci. Food Agr.* **25**, 607-612.
- OHTANI, S., OKADA, T., KAGAMIYAMA, H., AND YOSHIZUMI, H. (1975). The amino acid sequence of purothionin A, a lethal toxic protein for brewer's yeasts from wheat. *Agr. Biol. Chem.* **39**, 2269-2271.
- OKADA, T., AND YOSHIZUMI, H. (1973). The mode of action of toxic protein in wheat and barley on brewing yeast. *Agr. Biol. Chem.* **37**, 2289-2294.
- OKADA, T., YOSHIZUMI, H., AND TERASHIMA, Y. (1970). A lethal toxic substance for brewing yeast in wheat and barley: I. Assay of toxicity on various grains and sensitivity of various yeast strains. *Agr. Biol. Chem.* **34**, 1084-1088.
- REDMAN, D. G., AND FISHER, H. (1968). Fractionation and comparison of purothionin and globulin components of wheat. *J. Sci. Food Agr.* **19**, 651-658.
- RHEUBEN, M. B. (1972). The resting potential of moth muscle fiber. *J. Physiol.* **225**, 529-554.
- STUART, L. S., AND HARRIS, T. H. 1942. Bactericidal and fungicidal properties of a crystalline protein isolated from unbleached wheat flour. *Cereal Chem.* **19**, 288.