Improved Separation and Toxicity Analysis Methods for Purothionins[1]

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ABSTRACT

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A high-performance liquid chromatography (HPLC) method has been developed for separating α_1-, α_2-, and β-purothionins from bread wheat. The separation utilized C-18 reversed-phase columns and maximal resolution occurred at ice-water temperature. Up to 4 mg of an α_1- and α_2-purothionin mixture could be separated in one run, using a preparative column. Separation of a 4-mg sample yielded α_2-purothionin that was 97.4% pure, whereas HPLC separations of lesser amounts (up to 2 mg/run) gave essentially 100% pure material. β-Purothionin was completely separated from α_2-purothionin and was partially separated from α_1-purothionin by the HPLC method. A fast, reliable, and easily quantitated method for measuring the toxicity of purothionin to cultured mosquito and spruce budworm cells is reported. The test showed that cultured cells became more resistant to purothionin poisoning as they became older, and that 50% of fresh (three days old) budworm cells were killed by about 20 μg/ml of each of the three purothionin forms. Older budworm cells were less sensitive to β-purothionin than to either of the α-purothionins, indicating there may be some difference in the ways the α- and β-purothionins interact with the cells. Mosquito cells also showed LC_{50} values of about 20 μg/ml for all three purothionins. Subjecting α_1- and β-purothionins to HPLC separation conditions (pH 2.8, 7.3*M* acetonitrile) did not affect their toxicities to cultured cells.

Key words: Tissue culture, Toxicity analysis, Wheat

Purothionins are small proteins present in bread wheat (*Triticum aestivum* L. end. Thell.) (Balls et al 1942) and in various wheat relatives including diploid *Triticum* and *Aegilops* species (Carbonero and Garcia-Olmedo 1969). Homologous proteins, thionins, have been found in other cereals, including rye (Hernandez-Lucas et al 1978), barley (Redman and Fisher 1969), and oats (Békés and Lásztity 1981). They are of interest because their amino acid sequences have been used to study wheat evolution (Jones et al 1982) and because they affect various aspects of cell growth and function. Their in vivo functions in grain are not known, but they probably play an important role in the seed as they affect metabolism in several different ways. They apparently interact with cell walls to alter membrane permeabilities (Kramer et al 1979, Nakanishi et al 1979, Kashimoto et al 1979), specifically kill cultured cells during the DNA-synthetic phase of their growth (Nakanishi et al 1979), inhibit translation of mRNA into protein (Garcia-Olmedo et al 1983), and show thioredoxin activity (Wada and Buchanan 1981).

β-Purothionin is easily separated from either of the α-forms (α_1- and α_2-purothionins) by carboxymethylcellulose (CMC) ion-exchange chromatography (Redman and Fisher 1968). The two α-purothionins have been separated from each other by CMC chromatography (Jones and Mak 1977), but only partial separation could be obtained because both α-purothionins have the same net charge. Of the six differences between the α_1- and α_2-purothionin amino acid sequences, three are very conservative and probably have little effect on the characteristics of the molecules. The other three changes all have aliphatic amino acids (2 alanine, 1 glycine) of α_1-purothionin replaced by polar residues (2 serine, 1 threonine) in α_2-purothionin (Jones and Mak 1977). These changes should render α_1-purothionin more hydrophobic than α_2-purothionin. It therefore seemed reasonable to expect that high-performance liquid chromatography (HPLC) with C-18 reversed-phase columns, which separate molecules on the basis of hydrophobic interactions, might separate the two α-purothionin forms. Previous work (Jones and Lookhart 1985) has shown that pyridylethylated α_1- and α_2-purothionins could be separated by HPLC. This paper reports the separation of native α_1-, α_2-, and β-purothionins by HPLC.

Purothionin toxicity tests have been run on plants and animals ranging from bacteria and fungi (Stuart and Harris 1942) to insect larvae (Kramer et al 1979) and mammals (Coulson et al 1942). Tests with higher animals were hampered by the fact that thionins were not toxic when ingested but had to be injected into one small portion of the subject and thus were not well-distributed throughout the test animal. Recent investigations with cultured animal cells (Nakanishi et al 1979, Carrasco et al 1981) have shown they are sensitive to purothionin poisoning. A relatively easy and reliable method of determining the health and viability of cells in culture by measuring their adenosine triphosphate (ATP) content has been developed (Murphy et al 1976). The method has been used with cultured cells from the spruce budworm and mosquito to assay the toxicity of an entomocidal protein isolated from *Bacillus thuringiensis* (Johnson and Davidson 1984). This paper details how a modification of that method has been used to analyze the toxicity of native and HPLC-purified purothionins.

MATERIALS AND METHODS

Purothionin Isolation

Purothionins were isolated from bread flour (milled from *Triticum aestivum* cv. Manitou) and were prepared by CMC-ion exchange chromatography as described by Jones and Mak (1977) and Mak and Jones (1976a). Pure α_2-purothionin was prepared from *Aegilops squarrosa* L. (Jones and Lookhart 1985). Purity of the various purothionin preparations was analyzed by comparing the amino acid compositions of the fractions, as determined by HPLC of *o*-phthaldialdehyde amino acid derivatives of the hydrolyzed protein fractions (Lookhart et al 1982), with the expected compositions as calculated from the known amino acid sequences of the proteins (Jones and Mak 1977, Mak and Jones 1976b).

HPLC Separations

Protein samples containing about 0.5 μg/μl of purothionin dissolved in 15% acetonitrile (CH_3CN)/0.1% trifluoroacetic acid (TFA) solution were applied to a 4.6 mm × 25 cm SynChropak RP-P C-18 reversed-phase column (SynChrom, Inc., Linden, IN) via a Rheodyne injector valve. For preparative runs, 0.5 to 4 mg of purothionin was dissolved in 100 μl of 15% CH_3CN/0.1% TFA solution and applied to a 25 cm × 10 mm SynChropak RP-P column. The SynChropak RP-P columns are "macroporous," with 300 Å pores in their packing material. This allows small proteins

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and large polypeptides to more readily penetrate the matrix. A Varian 5060 pump was used to mix and pump the elution gradients. HPLC eluates were monitored at 210 nm with a Tracor 970 variable wavelength detector and the results were recorded on a Hewlett-Packard 3385A automation system. The compositions of gradients, solvent flow rates, and temperatures used to elute various purothionins are given in the Results section. Gradients were prepared by mixing a solution containing 80% CH$_3$CN in 0.1% TFA with one containing 15% CH$_3$CN in 0.1% TFA.

Toxicity Assay

Purothionin toxicities were measured by determining the effect of the protein upon the ATP content of cultured tissue cells. ATP is rapidly lost from cells that have been exposed to cytolytic toxins. The ATP remaining in living cells can be readily measured by determining the bioluminescence resulting when the cells are incubated with the luciferase-luciferin complex (Fig. 1). Bioluminescence is proportional to the concentration of ATP, for ATP concentrations of $10^{-6} M$ or less, when the cell concentration is $2–4 \times 10^5$ cells/ml.

The two insect cell lines used in this study were from the spruce budworm (*Choristoneura fumiferana* Clemens), FMPI-CF1, a suspended culture started from minced neonate larvae (Sohi 1973), and from the mosquito (*Anopheles gambiae* Pudney and Varma), an attached culture established from first instar larvae (Varma and Pudney 1969). Growth of FPMI-CF1 was on Grace's original tissue culture medium (Grace 1962), and *Anopheles gambiae* was grown on Singh's medium (Singh 1967); both media were supplemented with 15% inactivated fetal calf serum. The spruce budworm cells were grown at 28° C, and the mosquito cells were cultured at 25° C. Both were cultured in a stationary position in 25-cm^2 polystyrene culture flasks.

The ATP-luciferase-luciferin procedure used here for analyzing purothionin toxicity was essentially as reported by Johnson and Davidson (1984). Three- to five-day-old cells were collected by scraping with a rubber policeman and washed repeatedly by centrifugation in buffered insect saline (Murphy et al 1976). A final stock solution of cells ($2–3 \times 10^6$ cells/ml) was prepared for bioassay. Samples containing purothionin were diluted in buffered insect saline to a sequential series of protein concentrations ranging from 2 to 100 μg/ml, and 0.1 ml of each was added to 0.1 ml of stock cell suspension. Duplicate samples of each dilution series were included. Controls consisted of cells without purothionin and vice versa. Sample tubes containing cells and toxin were incubated at 30° C for 30 min, after which the reactions were terminated by the addition of 2 ml of boiling buffer (20 m*M* Tris-HCl, pH 7.0, 5 m*M* MgCl$_2$, 1 m*M* ethylenediamine tetraacetic acid). The incubation tubes were put into a boiling water bath for an additional 10 min to ensure complete lysis of the tissue cells. Upon cooling, the ATP content of each assay tube was measured with firefly luciferase (Sigma Chemical Company, St. Louis, MO) using a bioluminescent photometer (Lumac Biocounter model M2010, Lumac B.V., Schaesburg, Netherlands). A Commodore model 4032 computer (Commodore Business Machines, Norristown, PA) interfaced with the biocounter provided sample measurement and data analysis. All data were processed according to a program especially written to provide a least squares fit of the experimental data based upon a log protein/ml versus percent toxicity relationship.

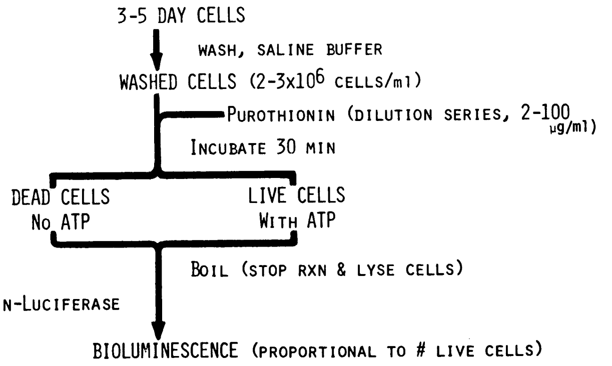

Fig. 1. Adenosine triphosphate bioluminescence analysis method used to measure toxicity of purothionins to cultured mosquito and spruce budworm cells.

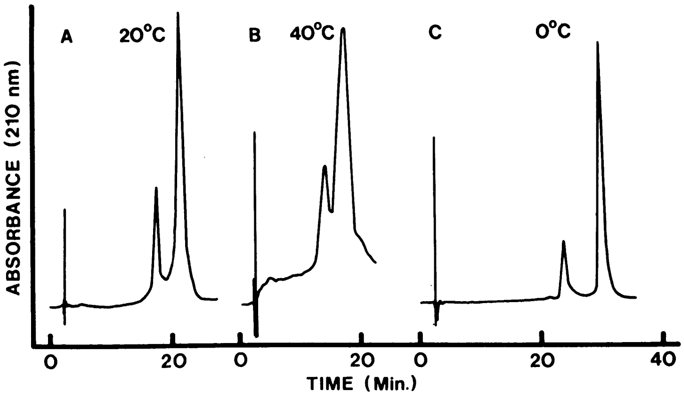

Fig. 2. Effect of temperature on high-performance liquid chromatography separation of α-purothionins.

RESULTS AND DISCUSSION

HPLC Separation of Purothionins

Previous experiments (Jones and Lookhart 1985) have shown that reduced and pyridylethylated α_1- and α_2-purothionins can be separated by HPLC using reversed-phase C-18 columns. Because native α_1- and α_2-purothionins are very difficult to separate by ion-exchange chromatography (Jones and Mak 1977), we applied a mixture of the two α-purothionins to a C-18 column and eluted them with an acetonitrile-TFA gradient.The α_1-, α_2-purothionin mixture analyzed was an "α_2-purothionin" preparation isolated from bread wheat by two successive passes through a CMC ion-exchange column. The preparation contained about 75% α_2-purothionin and 25% α_1-purothionin, as determined by amino acid analysis. About 10 μg of α-purothionin mixture was applied to and eluted from the HPLC column with the analytical gradient listed in Table I.

The overall elution gradient consisted of a series of three consecutive linear gradients running between the sets of conditions listed in Table I. For example, the analytical gradient started (time [t] = 0) at 16% B, and the concentration of B was increased by 0.25% per min until at t = 20 min, the eluant contained 21% B.

Using this gradient and an analytical HPLC column, the separation shown in Figure 2A was obtained when the column was eluted at 20° C. Separation was almost, but not quite, baseline. The α_1-purothionin eluted first (18 min), followed by α_2-purothionin (21 min). Elution order was determined by comparing the elution times of the two components with those of pure standard α_1- and

TABLE I
Gradients[a] Used to Elute Purothionins from C-18 Columns

Analytical Column[b]			Preparative Column[c]		
Time (min)	%B[d]	%CH$_3$CN	Time (min)	%B	%CH$_3$CN
0	16	25.4	0	16	25.4
20	21	28.7	1	20	28.0
25	25	31.3	20	25	31.3
26	16	25.4	21	16	25.4

[a] Overall gradient consisted of three consecutive linear gradients connecting the sets of conditions listed.

[b] 4.6 mm × 25 cm, flow rate = 1 ml/min.

[c] 10 mm × 25 cm, flow rate = 2 ml/min.

[d] Solvent B consisted of 80% CH$_3$CN + 0.1% trifluoroacetic acid (TFA). It was mixed with solvent A, 15% CH$_3$CN + 0.1% TFA, to form the gradients.

α_2-purothionins and by determining the amino acid compositions of the proteins in each peak. Separation was worse when the column was warmed to 40° C (Fig. 2B), but chromatography at 0° C resulted in a very good separation of the two α-purothionins (Fig. 2C), with a baseline period of more than 3 min between the times the two proteins eluted. Unlike small organic molecules, it is not unusual for proteins to separate more efficiently on HPLC at lower temperatures (Cohen et al 1984, Rivier 1978). It may be that, under the colder conditions, the purothionin molecules are "trapped" in more stable conformations, which would result in the narrowed elution bands observed (Cohen et al 1984). The α-purothionins eluted from the HPLC column in the reverse order from that expected. That is, the more hydrophilic α_2-purothionin form eluted after the hydrophobic α_1-form. Either the two purothionin forms must be assuming different conformations in solution such that different portions of the molecules are interacting with the packing material, or the separation is occurring via some mechanism other than by hydrophobic interactions. It doesn't seem likely that the two α-purothionins would assume significantly different conformations in solution because of their very similar primary structures, and because the secondary structures are probably tightly constrained by the large proportion of their amino acid residues that are intramolecularly disulfide-bonded cystine residues. In addition, results of Williams and Teeter (1984) have shown that α_1- and β-purothionins, and the much less homologous protein crambin, probably possess very similar structures in solution. It seems unlikely that the two α-purothionins, with much greater levels of homology, would possess very different secondary structures.

It seems at least equally likely that the separation of α_2-purothionin from the α_1- and β-forms in this system is caused, at least in part, by interactions between the proteins and silanol groups on the surface of the stationary phase. The SynChropak RP-P columns are not end capped, so that many free silanol groups would be present. The finding that α_2-purothionin binds to the column more strongly than α_1-purothionin would be explained by the fact that α_2-purothionin contains three hydroxyl-containing amino acids that are not present in α_1-purothionin. The three hydroxyl amino acids could hydrogen bond with the silanol groups to retard elution of the α_2-form. Finally, separation by silanol interactions is consistent with the fact that both α-purothionins are retained on the column longer at 0° C than at higher temperatures. Hydrogen bonding of the purothionins to silanol groups would be controlled by enthalpy changes, whereas hydrophobic interaction with the C-18 stationary phase would be dependent upon entropy changes. Hydrophobic (entropy) interactions would be expected to be stronger at higher temperatures (Elkoshi and Grushka 1981) but the hydrogen bonding (enthalpy driven) should be greater at lower temperatures. The stronger interactions of the purothionins with the column at ice temperatures implies they may be bound by enthalpy-driven forces.

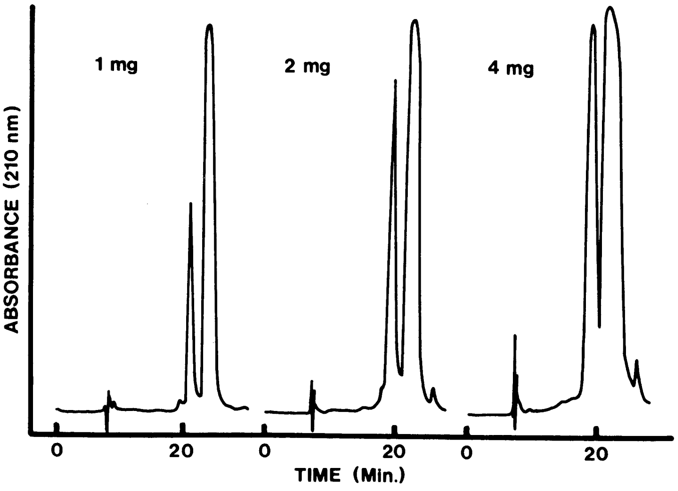

Fig. 3. Purification of α_2-purothionin by preparative high-performance liquid chromatography. Various quantities of an impure "α_2-purothionin" preparation from bread wheat, containing 74.2% α_2-purothionin and 25.8% α_1-purothionin, were applied to the column. The column was run in an ice-water bath.

To determine how much purothionin could be purified during a single HPLC run, the analytical column was replaced with a 10-mm diameter preparative column, and larger amounts of the protein mixture were applied. Figure 3 shows that when 1 mg of α-purothionin mixture was applied, nearly baseline separation was obtained. When the loading was increased to 2 mg, separation was not baseline, but purified α_1- and α_2-purothionin fractions were easily obtained. Application of 4 mg of protein still gave good separation, but the two peaks became so wide that totally pure thionins were hard to collect. When the material in the second (larger) peak from the 4 mg preparative run was collected and analyzed at 0° C on an analytical column (Fig. 4) it was seen that, whereas some α_1-purothionin (eluting at $T = 23$ min) was still present, the concentration had been reduced from 25.8% to 2.6% of the sample. For many purposes, the 97.4% pure α_2-preparation would be suitable. If not, and if one needed relatively large amounts of material, several 2 mg samples would have to be run consecutively through the column. With the 30-min turn-around time, even by running 2 mg samples one could purify at least 25 mg of α_2-purothionin per day. For many purposes, including toxicity analysis, amino acid analysis, and even amino acid sequence determination (Jones and Lookhart 1985), 5 mg of protein would be enough for several investigations.

β-Purothionin, which is readily separated from both α-purothionins by ion-exchange chromatography (Mak and Jones 1976b), can also be easily purified from α_2-purothionin by HPLC at 0° C (Fig. 5) and is partially separated from α_1-purothionin under the same conditions. The sample separated in Figure 5 was prepared by mixing pure α_1- and β-purothionins extracted from durum wheat with α_2-purothionin purified from *A. squarrosa*. It might be possible to completely purify the β- and α_1-purothionins from each other by modifying the gradient, solvent, or temperature conditions, but as we were only interested in purifying α_2-purothionin, we did not investigate that problem in any detail.

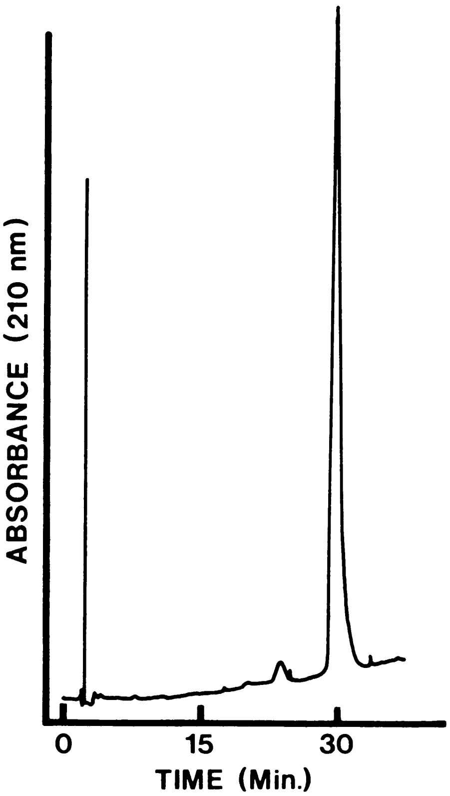

Fig. 4. Analytical high-performance liquid chromatography analysis of collected second (22 min) peak from the Figure 3, 4-mg separation.

When mosquito or spruce budworm cells were incubated with purothionins for 30 min, there was a linear relationship between the number of cells killed and the logarithm of the toxin concentration. This is shown in Figure 6, which illustrates the toxicity of native β-purothionin to spruce budworm cells. In this experiment the 50% lethal concentration of β-purothionin was 20.0 μg/ml. This value is somewhat higher than the 4–10 μg/ml that killed various cultured mammalian cells (Nakanishi et al 1979). Carrasco et al (1981) showed that translation of mRNA into protein in mouse and monkey cell lines was about 50% inhibited by 20 μg/ml purothionins, that protein synthesis in hamster cells was much more sensitive ($LC_{50} = < 1$ μg/ml) to purothionin and that in HeLa cells was less sensitive (20 μg/ml stopped only about 10% of the activity). The purothionin sensitivity of the Carrasco et al (1981) hamster cell protein synthesizing system was strongly dependent on the concentration of divalent metal ions, so their data are not directly comparable with ours.

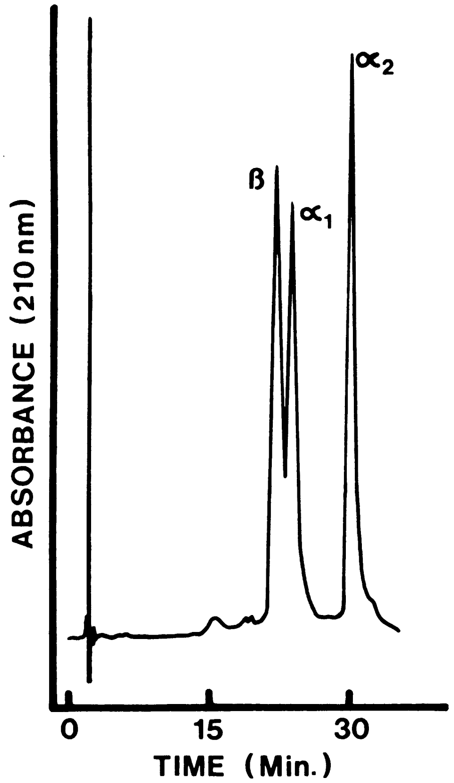

Fig. 5. Separation of the three bread wheat purothionins by high-performance liquid chromatography. α_1- and β-Purothionins were purified from durum wheat; α_2-purothionin was from *Aegilops squarrosa*.

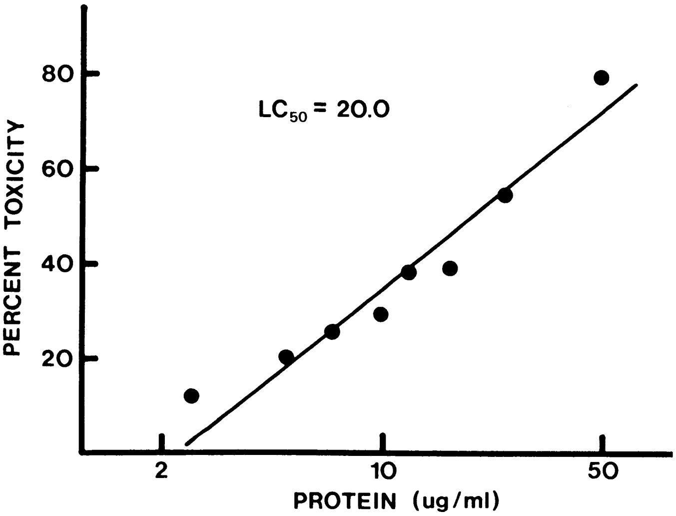

Fig. 6. Toxicity of β-purothionin to spruce budworm cells.

Garcia-Olmedo et al (1983) found that a cell-free wheat embryo protein synthesizing system was about 50% inhibited by approximately 25 μg/ml of purothionins, but the amount of inhibition was dependent upon the amount of RNA present in the system. Kramer et al (1979) demonstrated that the LD_{50} of β-purothionin injected into whole tobacco hornworm (*Manduca sexta*) larvae was 36 μg/g of larval weight, which is very similar to the results reported in this paper, considering that the protein injected into the hornworm may have remained partially compartmentalized in the larvae.

Toxicity tests with cells of varying ages showed that the cells became less sensitive to purothionin toxicity as they aged (Table II). The loss of sensitivity in older cells may be related to the fact that purothionins are much more toxic to animal cells during the period when DNA synthesis is occurring (the S-phase) than at other times (Nakanishi et al 1979). If, as seems likely, the insect cells used in this study multiply more slowly as they become older, then the older cells would be synthesizing DNA less often and should thus be less sensitive to purothionins.

With two- to four-day-old cells, both α_1- and β-purothionins were about equally toxic to the budworm cells and both purothionins were progressively less toxic as the cells became older. However, the resistance of the cells to poisoning by β-purothionin grew at a faster rate than did that to α_1-purothionin, and by the sixth day β-purothionin was only about half as toxic as α_1-purothionin. This result suggests that there is probably some difference in the way α_1- and β-purothionins affect the budworm cells. Because of this age-related toxicity variation, three-day-old cells were routinely used for all subsequent analyses.

Three-day-old cells of mosquito and spruce budworm were about equally sensitive to α_1-, α_2-, and β-purothionins (Table III). All six cell-purothionin combinations showed LC_{50} values of about

TABLE II
Effect of Cell Age on the Toxicity of Purothionins to Spruce Budworm Cells

Cell Age (days)	Purothionin LC_{50} (μg/ml)	
	α_1	β
2	11.7 ± 2.7[a]	11.9 ± 2.4
3	16.0 ± 4.0	16.7 ± 2.3
4	21.1 ± 3.9	20.5 ± 4.7
5	23.9 ± 5.6	29.8 ± 6.5
6	30.8 ± 8.7	58.8 ± 8.8

[a] 95% Confidence intervals.

TABLE III
Toxicities of the Different Purothionin Forms to Cultured Cells of Mosquito and Spruce Budworm

	Purothionin LC_{50} (μg/ml)		
Cell Line[a]	α_1	α_2	β
Mosquito	18.1 ± 4.4[b]	20.3 ± 3.8	19.5 ± 5.6
Spruce budworm	16.0 ± 4.0	25.3 ± 5.7	16.7 ± 2.3

[a] Three-day-old cell preparations.
[b] 95% Confidence intervals.

TABLE IV
Effect of High-Performance Liquid Chromatography (HPLC) Treatment on the Toxicity of Purothionins to Cultured Spruce Budworm Cells

	Purothionin LC_{50} (μg/ml)	
Purothionin	Native	HPLC Purified[a]
α_1	16.0 ± 4.0[b]	20.2 ± 6.9
β	16.7 ± 2.3	22.3 ± 6.3

[a] Subjected to pH 2.8 and 7.3*M* acetonitrile.
[b] 95% Confidence intervals.

19 μg/ml. From the data of Table II, however, it is apparent that the results would probably have been different if older cells had been treated and analyzed.

Table IV shows the effect of subjecting purothionins to the rigors of HPLC separation (exposure to low pH and to organic solvents). Purified α_1- and β-purothionins were tested for toxicity to spruce budworm cells, and, as expected for three-day-old cells, were about equally toxic. Samples of the tested α_1- and β-purothionins were dissolved in 15% CH_3CN/0.1% TFA solution and were adsorbed onto and eluted from a C-18 HPLC column at 0°C, using the preparative gradient of Table I. The purothionins were collected and freeze-dried, and their toxicities were analyzed. The data show that after HPLC the purothionins were as toxic to the cells as they were before being subjected to HPLC. Either the proteins were not altered during the HPLC process, or any changes that did occur did not affect their interaction with and toxicity to the cultured cells. It is possible that significant, but reversible, changes did occur in the structures of the proteins during HPLC separation, but that the proteins were renatured to their original conformations before they were analyzed. Under the same analysis conditions, reduced and pyridylethylated α_1- and β-purothionins did not kill any cells, even at concentrations of 100 μg/ml.

We have thus developed an HPLC method that allows total separation of multi-milligram amounts of α_1- and α_2-purothionins. We have also adapted a fast, reliable, and easily quantitated cultured-cell toxicity analysis method to testing purothionin toxicities. Finally, we have shown that little or no alteration in the structures of purothionins occurs during their separation by HPLC, since their toxicities are unaffected. Any structural changes that did occur would have to be either very minor or readily reversible.

LITERATURE CITED

BALLS, A. K., HALE, W. S., and HARRIS, T. H. 1942. A crystalline protein obtained from a lipoprotein of wheat flour. Cereal Chem. 19:279.

BÉKÉS, F., and LÁSZTITY, R. 1981. Isolation and determination of amino acid sequence of avenothionin, a new purothionin analogue from oat. Cereal Chem. 58:360.

CARBONERO, P., and GARCIA-OLMEDO, F. 1969. Purothionins in *Aegilops-Triticum* spp. Experientia 25:1110.

CARRASCO, L., VAZQUEZ, D., HERNANDEZ-LUCAS, C., CARBONERO, P., and GARCIA-OLMEDO, F. 1981. Thionins: plant peptides that modify membrane permeability in cultured mammalian cells. Eur. J. Biochem. 116:185.

COHEN, K. A., SCHELLENBERG, K., BENEDEK, K., KARGER, B. L., GREGO, B., and HEARN, M. T. W. 1984. Mobile-phase and temperature effects in the reversed phase chromatographic separation of proteins. Anal. Biochem. 140:223.

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.

ELKOSHI, Z., and GRUSHKA, E. 1981. Hydrophobic interactions and chromatographic processes. J. Phys. Chem. 85:2980.

GARCIA-OLMEDO, F., CARBONERO, P., HERNANDEZ-LUCAS, C., PAZ-ARES, J., VINCENTE, O., and SIERRA, J. M. 1983. Inhibition of eucaryotic cell-free protein synthesis by thionins from wheat endosperm. Biochim. Biophys. Acta 740:52.

GRACE, T. D. C. 1962. Establishment of four strains of cells from insect tissues grown *in vitro*. Nature 195:788.

HERNANDEZ-LUCAS, G., CARBONERO, P., and GARCIA-OLMEDO, F. 1978. Identification and purification of a purothionin homologue from rye. J. Agric. Food Chem. 26:794.

JOHNSON, D. E., and DAVIDSON, L. I. 1984. Specificity of cultured insect tissue cells for bioassay of entomocidal protein from *Bacillus thuringiensis*. In Vitro 20:66.

JONES, B. L., and LOOKHART, G. L. 1985. High performance liquid chromatographic separation of peptides for sequencing studies. Cereal Chem. 62:89.

JONES, B. L., and MAK, A. S. 1977. Amino acid sequences of two α-purothionins of hexaploid wheat. Cereal Chem. 54:511.

JONES, B. L., LOOKHART, G. L., MAK, A., and COOPER, D. B. 1982. Sequences of purothionins and their inheritance in diploid, tetraploid, and hexaploid wheats. J. Hered. 73:143.

KASHIMOTO, T., SAKAKIBARA, R., HUYNH, Q. K., WADA, H., and YOSHIZUMI, H. 1979. The effect of purothionin on bovine adrenal medullary cells. Res. Commun. Chem. Pathol. Pharmacol. 26:221.

KRAMER, K. J., KLASSEN, L. W., JONES, B. L., SPIERS, R. D., and KAMMER, A. E. 1979. Toxicity of purothionin and its homologues to the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae). Toxicol. Appl. Pharmacol. 48:179.

LOOKHART, G. L., JONES, B. L., COOPER, D. B., and HALL, S. B. 1982. A method for hydrolyzing and determining the amino acid compositions of picomole quantities of proteins in less than three hours. J. Biochem. Biophys. Methods 7:15.

MAK, A. S., and JONES, B. L. 1976a. Separation and characterization of chymotryptic peptides from α- and β-purothionins of wheat. J. Sci. Food Agric. 27:205.

MAK, A. S., and JONES, B. L. 1976b. The amino acid sequence of wheat β-purothionin. Can. J. Biochem. 54:835.

MURPHY, D. W., SOHI, S. S., and FAST, P. G. 1976. *Bacillus thuringiensis* enzyme-digested delta endotoxin: effect on cultured insect cells. Science 194:954.

NAKANISHI, T., YOSHIZUMI, H., TAHARA, S., HAKURA, A., and TOYOSHIMA, K. 1979. Cytotoxicity of purothionin-A on various animal cells. Gann 70:323.

REDMAN, D. G., and FISHER, N. 1968. Fractionation and comparison of purothionin and globulin components of wheat. J. Sci. Food Agric. 19:651.

REDMAN, D. G., and FISHER, N. 1969. Purothionin analogues from barley flour. J. Sci. Food Agric. 20:427.

RIVIER, J. E. 1978. Use of trialkyl ammonium phosphate (TAPP) buffers in reverse phase HPLC for high resolution and high recovery of peptides and proteins. J. Liquid Chromatogr. 1:343.

SINGH, K. R. P. 1967. Cell cultures from larvae of *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.). Curr. Sci. (India) 36:506.

SOHI, S. S. 1973. In vitro cultivation of larval tissues of *Choristoneura fumiferana* (Clemens) (Lepidoptera: Torticidae). Page 75 in: Proc. 3rd Int. Colloq. Invertebr. Tissue Culture. Publishing House of the Slovak Academy of Sciences: Smolenice, Bratislava, Czechoslovakia.

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.

VARMA, M. G. R., and PUDNEY, M. 1969. The growth and serial passage of cell lines from *Aedes aegypti* (L.) larvae in different media. J. Med. Entomol. 6:432.

WADA, K., and BUCHANAN, B. B. 1981. Purothionin: a seed protein with thioredoxin activity. FEBS Lett. 124:237.

WILLIAMS, R. W., and TEETER, M. M. 1984. Raman spectroscopy of homologous plant toxins. Crambin and α_1- and β-purothionin secondary structures, disulfide conformation, and tyrosine environment. Biochemistry 23:6796.

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