Improved Separation and Toxicity Analysis Methods for Purothionins

BERNE L. JONES, GEORGE L. LOOKHART, and DONOVAN E. JOHNSON

ABSTRACT

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 LC50 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, Nakaniishi et al. 1979, Kashimoto et al. 1979), specifically kill cultured cells during the DNA-synthetic phase of their growth (Nakaniishi et al. 1979), inhibit translation of mRNA into protein (Garcia-olmedo et al. 1983), and show thiorodoxin 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 α-phthalaldehyde 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 (CH₃CN)/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% CH₃CN/0.1% TFA solution and applied to a 25 mm × 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% CH₃CN in 0.1% TFA with one containing 15% CH₃CN 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 × 10⁴ cells/ml.

The two insect cell lines used in this study were from the spruce budworm (Choristoneura fumiferana Clemens), FPMI-CF1, a suspended culture started from mired neonate larvae (Sohi 1973), and from the mosquito (Anopheles gambiae) Pudney and Varma, an anachronous 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-cm² 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 × 10⁶ 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 mM Tris-HCl, pH 7.0, 5 mM MgCl₂, 1 mM 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., Schaeburg, 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.

RESULTS AND DISCUSSION

HPLC Separation of Purothionins

Previous experiments (Jones and Lookhart 1985) have shown that reduced and pyridylethylated α₃- and α₂-purothionins can be separated by HPLC using reversed-phase C-18 columns. Because native α₃- and α₂-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 α₃-, α₂-purothionin mixture analyzed was an α₂-purothionin preparation isolated from bread wheat by two successive passes through a CMC ion-exchange column. The preparation contained about 75% α₂-purothionin and 25% α₁-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 1.

The overall elution gradient consisted of a series of three consecutive linear gradients running between the sets of conditions listed in Table 1. 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 α₁-purothionin eluted first (18 min), followed by α₂-purothionin (21 min). Elution order was determined by comparing the elution times of the two components with those of pure standard α₁- and

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**Table 1**

<table>
<thead>
<tr>
<th>Gradients Used to Elute Purothionins from C-18 Columns</th>
<th>Analytical Column</th>
<th>Preparative Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>%B</td>
<td>%CH₃CN</td>
</tr>
<tr>
<td>0</td>
<td>16</td>
<td>25.4</td>
</tr>
<tr>
<td>20</td>
<td>21</td>
<td>28.7</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>31.3</td>
</tr>
<tr>
<td>26</td>
<td>16</td>
<td>25.4</td>
</tr>
</tbody>
</table>

*Overall gradient consisted of three consecutive linear gradients connecting the sets of conditions listed.

*4.6 mm × 25 cm, flow rate = 1 ml/min.

*10 mm × 25 cm, flow rate = 2 ml/min.

*Solvent B consisted of 80% CH₃CN + 0.1% trifluoroacetic acid (TFA). It was mixed with solvent A, 15% CH₃CN + 0.1% TFA, to form the gradients.

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Fig. 1. Adenine 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.

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CEREAL CHEMISTRY
α2-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 cooler 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 α1-purothionin form eluted after the hydrophobic α2-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 α1- 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 α2-purothionin from the α1- 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 α2-purothionin binds to the column more strongly than α1-purothionin would be explained by the fact that α2-purothionin contains three hydroxyl-containing amino acids that are not present in α1-purothionin. The three hydroxyl amino acids could hydrogen bond with the silanol groups to retard elution of the α2-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.

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 α2-purothionin mixture was applied, nearly baseline separation was obtained. When the loading was increased to 2 mg, separation was not baseline, but purified α1- and α2-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 α1-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 α2-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 α2-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 α2-purothionin by HPLC at 0°C (Fig. 5) and is partially separated from α1-purothionin under the same conditions. The sample separated in Figure 5 was prepared by mixing pure α1- and β-purothionins extracted from durum wheat with α2-purothionin purified from A. squarrosa. It might be possible to completely purify the β- and α2-purothionins from each other by modifying the gradient, solvent, or temperature conditions, but as we were only interested in purifying α2-purothionin, we did not investigate that problem in any detail.

![Figure 3](image-url) Fig. 3. Purification of α2-purothionin by preparative high-performance liquid chromatography. Various quantities of an impure "α2-purothionin" preparation from bread wheat, containing 74.2% α2-purothionin and 25.8% α1-purothionin, were applied to the column. The column was run in an ice-water bath.

![Figure 4](image-url) 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 purothionin 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 $\beta$-purothionin to spruce budworm cells. In this experiment the 50% lethal concentration of $\beta$-purothionin was 20.0 $\mu$g/ml. This value is somewhat higher than the 4–10 $\mu$g/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 $\mu$g/ml purothionins, that protein synthesis in hamster cells was much more sensitive ($L_{C50} = 1.2 \mu$g/ml) to purothionin and that in HeLa cells was less sensitive (20 $\mu$g/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.

Garcia-Olmedo et al. (1983) found that a cell-free wheat embryo protein synthesizing system was about 50% inhibited by approximately 25 $\mu$g/ml of purothionin, but the amount of inhibition was dependent upon the amount of RNA present in the system. Kramer et al. (1979) demonstrated that the $L_{D50}$ of $\beta$-purothionin injected into whole tobacco hornworm (Manduca sexta) larvae was 36 $\mu$g/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 larve.

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 $\alpha_1$ and $\beta$-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 $\beta$-purothionin grew at a faster rate than did that to $\alpha_1$-purothionin, and by the sixth day $\beta$-purothionin was only about half as toxic as $\alpha_1$-purothionin. This result suggests that there is probably some difference in the way $\alpha_1$- and $\beta$-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 $\alpha_1$, $\alpha_2$, and $\beta$-purothionins (Table III). All six cell-purothionin combinations showed $L_{C50}$ values of about

![Fig. 5. Separation of the three bread wheat purothionins by high-performance liquid chromatography. $\alpha_1$- and $\beta$-Purothionins were purified from durum wheat; $\alpha_1$-purothionin was from Aegilops squarrosa.]

![Fig. 6. Toxicity of $\beta$-purothionin to spruce budworm cells.]

<table>
<thead>
<tr>
<th>Cell Age (days)</th>
<th>$\alpha_1$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>11.7 ± 2.7</td>
<td>11.9 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>16.0 ± 4.0</td>
<td>16.7 ± 2.3</td>
</tr>
<tr>
<td>4</td>
<td>21.1 ± 3.9</td>
<td>20.5 ± 4.7</td>
</tr>
<tr>
<td>5</td>
<td>23.9 ± 5.6</td>
<td>29.8 ± 6.5</td>
</tr>
<tr>
<td>6</td>
<td>30.8 ± 8.7</td>
<td>58.8 ± 8.8</td>
</tr>
</tbody>
</table>

*95% Confidence intervals.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>$\alpha_1$</th>
<th>$\alpha_2$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mosquito</td>
<td>18.1 ± 4.4</td>
<td>20.3 ± 3.8</td>
<td>19.5 ± 5.6</td>
</tr>
<tr>
<td>Spruce budworm</td>
<td>16.0 ± 4.0</td>
<td>25.3 ± 5.7</td>
<td>16.7 ± 2.3</td>
</tr>
</tbody>
</table>

*Three-day-old cell preparations.

*95% Confidence intervals.

<table>
<thead>
<tr>
<th>Purothionin</th>
<th>LC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>HPLC Purified*</td>
</tr>
<tr>
<td>$\alpha_1$</td>
<td>16.0 ± 4.0</td>
</tr>
<tr>
<td>$\beta$</td>
<td>16.7 ± 2.3</td>
</tr>
</tbody>
</table>

*Subjected to pH 2.8 and 7.3 M acetonitrile.

*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- 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- and β-purothionins were dissolved in 15% CH3CN/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 subject 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- 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- and α-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


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