High Performance Liquid Chromatographic Separation of Peptides for Sequencing Studies

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ABSTRACT

High performance liquid chromatography (HPLC) has proven valuable in preparing, separating, and analyzing peptides for amino acid sequencing studies. The method is especially useful in instances where only small amounts of protein are available for sequencing. This paper reports several ways we have used HPLC peptide separations while determining the amino acid sequences of thionin proteins isolated from diploid wheat relatives. HPLC was used to confirm the purity of the thionins prior to sequencing, to separate thionins from each other, and to monitor the progress of enzymatic protein hydrolysis reactions to ensure that hydrolysis was complete. The method was used to analyze the proteolytic peptide “fingerprints” of thionins purified from bread wheat and from wheat relatives. This gave an indication of which of the bread wheat thionins was most similar (or identical) to that isolated from the diploid relative. Finally, HPLC was used to separate enzymatic hydrolysates of purothionins into pure peptides that were collected for further analysis.

High performance liquid chromatography (HPLC) is rapidly becoming the method of choice for studying, separating, and purifying proteins and peptides (see review by Regnier and Gooding [1980] and book by Lottspeich et al [1981]). Both size exclusion (Bietz 1984) and reversed-phase (Bietz 1983, 1985) HPLC methods have been used to examine cereal storage proteins, but to date HPLC has not been applied to the study of peptides from cereal grains.

We have been investigating the amino acid sequences of purothionin proteins from various wheat (Triticum) and Aegilops species to determine how the genomes from diploid wheat progenitor species have been incorporated into tetraploid durum wheat and hexaploid bread wheat (T. aestivum) (Jones et al 1982). The purothionins proteins are appropriate for such studies because they are small (relatively easy to sequence) (Balls et al 1942), easily purified (Mak and Jones 1976), and are widely distributed among the wheat species (Carbonero and Garcia-Olmedo 1969).

Relatively large amounts (approximately 100 mg) of the purothionins from T. aestivum, T. durum, and T. monococcum were available for sequencing, because these species are commercially available or easily propagated (Mak and Jones 1976a; Jones and Mak 1977, 1983). However, most of the alleged wheat progenitor species (diploid Triticum and Aegilops species) are grasses that are difficult to grow and that yield very small amounts of endosperm material when milled. This precluded our obtaining enough purothionin material from the diploids for sequencing by the standard Edman methodology (Edman and Begg 1967). Instead, we prepared nested sets of proteolytic peptides from purothionins extracted from the progenitor species, separated the peptides, and analyzed their amino acid compositions. This method allowed us to deduce the amino acid sequences of purothionins that were available in milligram amounts (Jones et al 1981). We found reversed-phase HPLC a most successful method for obtaining purothionin peptides for analysis. This paper reports the various HPLC methodologies we have used in our studies.

MATERIALS AND METHODS

Purothionin Isolation and Alkylation

Purothionins were isolated from various Triticum and Aegilops species by acid extraction and ion-exchange purification. Whole cereal seeds (200 g) were ground in a Udy cyclone mill to pass a 1-mm screen. The ground grain was suspended in 1 L of 0.05 M H2SO4, stirred well, and allowed to sit for 2 hr at room temperature, with occasional stirring. The pH of the suspension was below 3.0. The slurry was then centrifuged at 27,000 ×g (15,000 rpm), at 15°C, for 20 min in a Sorvall SS-34 rotor. The centrifuged precipitate was resuspended in 750 ml of 0.05 M H2SO4, incubated at room temperature for 1 hr, and centrifuged as before. The two 15,000 rpm supernatants were combined and adjusted to pH 8.0 with concentrated NH4OH.

At pH 8.0, the solution turned from green to yellowish. The pH 8.0 solution sat for 2 hr at room temperature and was then centrifuged at 27,000 ×g for 20 min. The supernatant was adjusted to pH 5.2 with glacial acetic acid and allowed to stand for 1 hr at room temperature. If a haze formed, it was removed by centrifuging at 27,000 ×g.

The clarified solution was pumped onto a 2.5 × 15-cm carboxymethyl cellulose (CMC, Whatman CM 52 preswollen microgranular) column that had been equilibrated with 0.1 M ammonium acetate (NH4Ac), pH 5.2. The loaded column was washed with 0.1 M NH4Ac, pH 5.2, until no more 280-nm-absorbing material eluted. The column was developed with a 0.3–1.5 M linear gradient of NH4Ac, pH 5.2, using 500 ml of each concentration buffer. Protein elution was determined by monitoring the absorbance of the eluent at 280 nm. Proteins that eluted at salt concentrations greater than 0.6 M were tested for precipitation in the presence of sodium dodecyl sulfate (SDS). Precipitation in the presence of SDS is characteristic of thionins (B. L. Jones, unpublished). Peaks containing material that precipitated in the presence of SDS were collected, diluted twofold with water, and applied to a 1.2 × 15-cm CMC column equilibrated with 0.1 M NH4Ac, pH 5.2. In all cases, the SDS-precipitating protein was the last protein eluted from the CMC column. The 1.2-cm column was eluted with a 0.3–1.2 M linear NH4Ac gradient, pH 5.2 (250 ml each buffer), and column effluent was monitored at 280 nm. When symmetrical peaks were obtained, the 280-nm-absorbing tubes were pooled and freeze-dried twice to remove NH4Ac. If the peaks were only partially separated, the tubes were pooled so that maximum separation was retained, and the partially purified fractions were rechromatographed until symmetrical peaks were obtained.

Purothionins were reduced and pyridylethylated whenever appropriate by the method of Mak and Jones (1976a), except that desalting was accomplished by applying the reaction mixture to a small CMC column (equilibrated with 0.1 M NH4Ac, pH 5.2), washing with 0.1 M NH4Ac until all nonbound 254-nm-absorbing material eluted, and then developing the column with a 0.3–1.2 M gradient of NH4Ac. The 254-nm-absorbing peak that eluted at about 0.9 M NH4Ac was collected, freeze-dried twice, and weighed.
Enzymatic Hydrolyses

Three site-specific protease enzymes were used to prepare nested sets of peptides from pyridylethylated purothionins.

Trypsin hydrolysis. Pyridylethylated purothionin (0.12 mg) was weighed into a 1.5-ml polyethylene conical centrifuge tube and suspended in 50 µl of PIPES (piperazine-N,N'-bis[2-ethane sulfonyl acid], Sigma Chemical Co.) buffer (100 mM, pH adjusted to 6.8 with NaOH). Ten microliters of enzyme solution (0.1 mg/ml of trypsin [ Worthington, lot-1-Tosylamido-2-phenyl] ethyl chloromethyl ketone (TPCK)-treated) dissolved in PIPES buffer) was added to the mixture, stirred well, and incubated at 37°C. Samples were removed at appropriate times, frozen in a dry ice bath, and freeze-dried for storage until analyzed.

Endoproteinase Lys-C (EPLC) hydrolysis. A sample of EPLC enzyme from Boehringer-Mannheim was dissolved in distilled water to a concentration of 0.2 mg/ml and frozen. Whenever reaction mixtures were prepared, the enzyme solution was thawed, an aliquot removed, and the remaining solution was refrozen.

Reduced and pyridylethylated purothionin (0.1 mg) was dissolved in 30 µl of 1 mM EDTA and 25 mM Tris (adjusted to pH 7.7 with HCl) buffer in a 1.5-ml polyethylene centrifuge tube. EPLC enzyme solution (2.5 µl) was mixed with the substrate solution and incubated at 38°C. At appropriate times, samples were removed, freeze-dried, and stored for analysis.

Clostripain hydrolysis. Reduced and pyridylethylated purothionin (0.1 mg) was dissolved in 50 µl of 20 mM Tris HCl, pH 7.4, buffer. An enzyme solution was prepared that consisted of 90 µg clostripain (Boehringer-Mannheim), 73 mg CaCl2, and 0.8 mg of dithiothreitol dissolved in 5 ml of H2O. Enzyme solution (50 µl) was added to the purothionin solution, mixed well, and the solution was incubated at 38°C for appropriate periods of time. The reaction was terminated by freezing and freeze-drying the reaction mixture.

Analysis of Peptides by HPLC

For analysis, freeze-dried, enzymatically hydrolyzed protein samples containing about 0.10 mg of hydrolyzed protein were dissolved in 50 µl of 0.10% trifluoroacetic acid (TFA) solution. The samples were applied to a 4.6 mm × 30-cm Synchronpak RP-P column (Synchron, Inc., Linden, IN) via a Rheodyne injector valve fitted with a 100-µl sample loop. A Varian 5060 pump was used to reproducibly mix and pump the elution gradient. HPLC eluates were monitored at 220 nm with a Tracor 970 variable wavelength detector and at 280 nm with an Isco UAS detector. The samples were pumped through the ultraviolet detectors in series so that the absorbances of all peptide separations were recorded at both 220 and 280 nm. The outputs from the Tracor and Isco detectors were recorded and analyzed with Hewlett-Packard 3388A and 3385A automation systems, respectively. Peptides were eluted with a 0.1% TFA-acetonitrile gradient: its composition is given in Results and Discussion. HPLC-separated peptide fractions were collected in test tubes, dried under vacuum, and stored until analyzed. HCl (6N) hydrolyses were conducted under vacuum, at 155°C as reported earlier (Lookhart et al 1982). Amino acid compositions were determined by HPLC analysis of amino acid—o-phthalaldehyde derivatives as reported by Lookhart et al (1982).

RESULTS AND DISCUSSION

The purothionins are small (45 amino acid residues) proteins found in wheat seeds (Ball et al 1942). We are using the amino acid sequences of these proteins to delineate the evolution of wheats from diploid progenitor species through to hexaploid bread wheat (Jones et al 1982). The amino acid sequences of the three purothionin species present in T. aestivum (bread wheat) are shown in Figure 1. Only those residues of α- and α- purothionins that differ from β-purothionin are listed. Figure 1 also shows the points at which three proteolytic enzymes used in this study should normally hydrolyze β-purothionin. All three proteases hydrolyze peptide bonds adjacent to (on the C-terminal side of) amino acids that have basic side chains (arginine R, and lysine K). Trypsin hydrolyzes bonds adjacent to both arginine and lysine, whereas EPLC hydrolyzes only those next to lysine, vacuolins with optimal conditions attacks those beside arginine. Trypsin does not normally hydrolyze bonds adjacent to lysine residues that occur at the N-termini of proteins, so no cleavage point has been indicated between residues 1 and 2. The only amino acid differences between α- and β-purothionins that involve basic amino acid residues are at residues 5, where the lysine of β-purothionin (amino acid EPLC hydrolysis) is replaced with arginine (amino acid EPLC hydrolysis, resistant to EPLC) in both α-purothionins. Complete hydrolysis of any of the purothionins with trypsin should release nine peptides (or 10 if cleavage occurred between residues 1 and 2), whereas EPLC should hydrolyze β-purothionin into six peptides and the α-purothionins into five. Clostripain hydrolysis should form five (β-) or six (α- and α-) purothionin peptides.

The HPLC program shown in Figure 2 gave maximal separations of the purothionin proteolytic peptides examined. It separated all peptides, except that single residues of lysine and some very small peptides did not bind to the column but eluted together at the void volume. To ensure good reproducibility of the HPLC peptide separations, a 25-min equilibration period was allowed between the time one run finished and when the next sample was injected. This gave a total per-analysis turnover time of about 65 min. The column was equilibrated for 1 hr with 2% acetonitrile (CH3CN) each morning, before the first sample was injected. If the column had not been used for two or more days, it was conditioned before the first peptide mixture was applied by

Fig. 1. Amino acid sequences and protease hydrolysis positions of Triticum aestivum purothionins. The complete sequence is that of β-purothionin. Only amino acids differing from β-purothionin are listed for α- and α- purothionins. Each letter represents one amino acid, using the terminology of Hunt et al (1976). Arrows indicate where β-purothionin is most susceptible to hydrolysis by: ⊣, trypsin; ⊣, endoproteinase Lys-C (EPLC); and ⊣, clostripain.

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pumping 2% acetonitrile through the column for 30 min and then running through a complete elution cycle without applying any sample.

**Use of HPLC to Isolate and Determine the Purity of Substrates**

Before starting to sequence a protein, it is mandatory that it be free of contamination by other proteins. One way to check the purity of the protein is to see whether it elutes from an HPLC column as a single entity. This can only be done, of course, if the protein is soluble in the solution used to develop the HPLC column. Since the purothionins have molecular weights of about 5,000, they may be considered very large peptides. They are soluble in the TFA-CH₂CN gradients used to elute their proteolytic peptides and can be analyzed with the program shown in Figure 2. Figure 3A shows the chromatogram obtained when a sample of reduced and pyridylethylated purothionin (PE purothionin) extracted from *T. monococccum* was analyzed by HPLC. *T. monococccum* contains a single purothionin protein, which has been shown by Jones and Mak (1983) to have an amino acid sequence identical to that of β-purothionin from *T. aestivum*. The single large peak shows that the sample is essentially pure. Figure 3B shows that PE α₁- and PE α₂-purothionins are readily separated from each other by HPLC. It is possible to separate the α₁- and α₂-purothionins using ion-exchange chromatography (Jones and Mak 1977), but the separation is poor, and the method is very time consuming. The HPLC separation is clearly the method of choice for separating the two α-purothionins, if only small amounts are needed, and if they can (or must) be reduced and alkylated.

While β-purothionin is readily separated from both α-purothionins by ion-exchange chromatography (Redman and Fisher 1968), this HPLC method did not separate PE α₂- and PE β-forms.

**Using HPLC to Monitor the Progress of Hydrolysis**

When proteolytic peptides are prepared for sequence analysis, it is necessary to know how long to allow hydrolysis to continue in order to obtain appropriate peptides. For initial studies, it is normally best to incubate with the proteolytic enzyme until all of the readily susceptible bonds have been cleaved, but not so long that nonspecific bond scission can occur. These hydrolysis conditions generally lead to the most easily separated peptide mixtures, and the peptides so obtained usually give the most easily interpreted sequence data. If, on the other hand, one needs to obtain overlapping peptides to align the peptides in the original protein, it is often advantageous to stop the hydrolysis reaction before all of the readily hydrolyzable bonds have been broken.

Figure 4 shows results obtained when PE β-purothionin was hydrolyzed with EPLC enzyme at a 200:1 (w/w) purothionin:enzyme ratio. Aliquots of the reaction mixture were removed after incubation times from 0 to 240 min, but only the fractions removed at or prior to 30 min of reaction are shown here. At time (t) = 0, no hydrolysis had taken place. The peak at t = 2.3 min is an artifact caused by one of the components of the reaction mixture that elutes in the void volume and absorbs strongly at 280 nm. The single peak at 40 min is whole PE β-purothionin. After incubation for 1 min, hydrolysis had begun, as the amount of whole protein was reduced and several smaller peptides had formed. In general, the smaller peptides elute more quickly than do larger ones.

After 3 min of incubation, well over half of the original protein was gone; after 15 min, nearly all had been destroyed. Within 30 min, the enzyme had completely converted the protein into relatively small peptides, and the late-eluting large peptides were gone. From these data it is apparent that if one wanted larger "overlap" peptides—to get information about which small peptides

![Fig. 2](image-url)  
**Fig. 2.** High performance liquid chromatography program used to separate pyridylethylated purothionins and proteolytic peptides of purothionins. The program consisted of five consecutive linear gradients. Acetonitrile was mixed with a 0.10% trifluoroacetic acid solution to prepare the required gradients.

![Fig. 3](image-url)  
**Fig. 3.** Analysis of pyridylethylated purothionin preparations by high performance liquid chromatography. A, analysis of pyridylethylated β-purothionin. B, analysis of a mixture of pyridylethylated α₁- and α₂-purothionin preparations.
occupy adjacent positions in the protein—he would collect and characterize those peptides from the 3- and 15-min hydrolysates which elute between t = 33 and 43 min. A better quantitative distribution of these fractions would probably have been obtained if an 8- or 10-min hydrolysis fraction had been collected.

The enzyme EPLC was very specific in hydrolyzing PE β-puromethionin only at lysine residues. This is seen from Figure 5A, which shows the peptide pattern obtained when a 2-hr hydrolysate was analyzed. The pattern was essentially identical with that of the 30-min hydrolysate (Fig. 4), with the same four peaks clearly predominating. From Figure 1 it is apparent that hydrolyzing β-puromethionin at all lysine residues should have resulted in the release of five peptides. Only four peaks are present in Figure 5A, however, because when the absorbance at 280 nm is monitored, only those peptides containing pyridylethyl cysteine (PECys) or tyrosine residues were detected. Because the EPLC peptide covering residues 42-45 contained neither of those amino acids, it did not absorb at 280 nm. Figure 5B, showing the same analysis monitored at 220 nm, revealed five peptides (in addition to the t = 2.3 min artifact). Since all peptides absorb light at 220 nm, the peak at t = 17.0 min must have represented the peptide consisting of amino acid residues 42 through 45. The areas under the individual peaks of the 280-nm chromatogram are approximately proportional to the number of residues of PECys + tyrosine present in each. This, together with the knowledge that small peptides elute before larger ones, allowed certain predictions about which peaks correspond to given peptides. For example, from Figure 5A, the peptides, in order of elution, probably contained 2, 3, 1, and 3 residues of (PECys + tyrosine) respectively, since whole β-puromethionin contains 8 cysteine and 1 tyrosine residues. The first peptide fraction probably contained residues 1-5 (or 2-5, if EPLC removed the N-terminal lysine), as it had to be small and contained two residues of PECys. Likewise, the t = 23 min peptide probably encompassed residues 24-32 (medium size, 3 PECys residues) and the t = 32 min peptide (large, 2 PECys, 1 tyrosine) probably ran from residue 6 through 23.

Not all proteolytic enzymes gave results as clearly defined as those from EPLC. When a β-puromethionin sample was hydrolyzed with clostripain (at a 1:110 w/w, enzyme:substrate ratio), hydrolysis was rapid, with the expected five peptides present after only 8 min (Fig. 6). Hydrolysis by this clostripain preparation was not entirely specific for arginine residues, because small extra peptide peaks showed up at t = 10.3 and 22.3 min. After 1 hr incubation, 10 peptides absorbing at 280 nm were released from the β-puromethionin (Fig. 6). No further sites on the protein were readily susceptible to clostripain hydrolysis, because no new major peptides were released between 1 and 4 hr of reaction. Yet, during that time, the areas of the various peptide peaks changed as hydrolysis of susceptible residues proceeded toward completion.

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**Fig. 4.** High performance liquid chromatography separations of peptides formed by the hydrolysis of pyridylethylated β-puromethionin by endoproteinase Lys-C (EPLC) enzyme. Effect of hydrolysis time on elution patterns.

**Fig. 5.** High performance liquid chromatography separation of peptides from a 2-hr hydrolysate of pyridylethylated β-puromethionin with endoproteinase Lys-C (EPLC) enzyme. A, absorbance of eluate monitored at 280 nm. B, absorbance of eluate monitored at 220 nm.
Figure 6 thus illustrates why it is important to monitor the progress of enzymatic hydrolyses: if larger peptides arising from primary hydrolysis cleavages were needed for sequence studies, the optimum reaction time would be 8 min. If, however, small peptides, including those arising from secondary, less specific, hydrolyses were required, hydrolysis should be allowed to proceed for 4 hr. In addition, by removing half of the mixture at 8 min and allowing the other half to react 4 hr, and then comparing the compositions of the 8-min and 4-hr peptides, one could obtain some information about which of the smaller 4-hr peptides were adjacent in the original protein molecule. An example of this is discussed later.

**Use of HPLC Peptide Patterns for “Fingerprinting” Proteins**

Proteins can be hydrolyzed with proteases that are specific for given amino acid residues, and the resulting peptide mixture can be separated by HPLC to give a peptide elution profile or “fingerprint” pattern that is characteristic of the protein being hydrolyzed and of the protease used to hydrolyze it. Figure 7A–C shows the elution patterns obtained when β-, α1-, and α2-purothionins were hydrolyzed for 1.5 hr with EPLC enzyme and the resulting peptides were separated by HPLC. The three purothionins each broke down into sets of peptides that yielded distinct peptide HPLC elution patterns. When a thionin extracted from the diploid species *Aegilops squarrosa* was hydrolyzed under the same conditions, it showed a peptide-elution pattern (Fig. 7D) identical to that of α2-purothionin, an indication that the *A. squarrosa* thionin probably has a primary structure identical with that of α2-purothionin. Complete peptide analysis of the *A. squarrosa* thionin has shown that it is identical to that of *T. aestivum* α2-purothionin (Jones et al 1981). HPLC peptide elution patterns obtained with peptides prepared by trypsin digestions of different purothionins are shown in Figure 8. The α1-, α2-, and β-purothionins all give fingerprint patterns that are distinctly

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**Fig. 6.** High performance liquid chromatography separations of peptides formed by the hydrolysis of pyridylethylated β-purothionin by clostripain. Effect of hydrolysis time on elution patterns.

**Fig. 7.** High performance liquid chromatography peptide elution patterns of hydrolysates prepared by hydrolyzing various pyridylethylated purothionins with endoproteinase Lys-C (EPLC) enzyme for 1.5 hr.
different from each other. Patterns of peptides obtained from tryptic digests of purothionins isolated from *T. monococcum* (Fig. 8D) and *T. urartu* (Fig. 8E) were identical with each other and were the same as that of β-purothionin from *T. aestivum*. Each consisted of a large peak at t = 7.4 min, with smaller flanking peaks; three large peaks at t = 21.0, 22.3, and 23.6 min; and finally a small peak at 27.7 min. This is consistent with the facts that both *T. monococcum* (Jones and Mak 1983) and *T. urartu* (Jones et al. 1981) have been shown to contain thionins identical with *T. aestivum* β-purothionin.

**Separation of Peptides for Further Studies**

While peptide fingerprint patterns of proteins can provide evidence of protein structural identity or homology, it is often necessary to further characterize the peptides by determining their amino acid compositions or even their amino acid sequences. Because of the speed of separation, the exceptional intrinsic resolving power, and the sensitivity of detection of HPLC, it is an ideal method of separating peptide mixtures for analytical studies.

The following example demonstrates one such use. From Figure 6 it was obvious that clostripain probably hydrolyzed β-purothionin at residues other than arginine when the enzyme and substrate were incubated together for more than about 10 min. It has been reported that clostripain hydrolyzes peptide bonds adjacent to lysine residues at a rate of approximately 1/10–1/100 that of bonds next to arginine (Mitchell and Harrington 1971). To ascertain where β-purothionin was cleaved by clostripain, hydrolysis was continued for 4 hr, and the resulting peptide mixture was separated by HPLC (Fig. 9B). Comparison of the 8-min (Fig. 9A) and 4-hr hydrolysates showed that all of the peptides present at 8 min (peptides 3, 6, 8, 9, and 11) were still found in the 4-hr hydrolysate, but all except peptide 3 were present in reduced amounts. All 280-nm-absorbing fractions were individually collected (as indicated in Fig. 9B), dried under vacuum, hydrolyzed with 6N HCl, and their amino acid compositions were determined by HPLC after reaction with o-phthaldehyde (Lookhart et al. 1982). This method is sensitive enough that less than 3% of the HPLC-separated peptide isolated from the hydrolysate of 0.10 mg of purothionin was needed for analysis. Figure 10 shows the amino acid analysis of an aliquot of HCl-hydrolyzed peptide fraction 7 from Figure 9B. The peptide was hydrolyzed with HCl(6N, 155°C, 60 min) using the method of Lookhart et al. (1982); 7.2% of the hydrolyzed sample was reacted with o-phthalaldehyde, and the amino acid derivatives were separated by HPLC and quantitated.

**ELUTION PATTERNS OF PUROTHIONIN TRYPTIC PEPTIDES**

![Fig. 8. High performance liquid chromatography peptide elution patterns of hydrolysates prepared by hydrolyzing various pyridylethylated purothionins with trypsin. A–C, α1–α3, and β-purothionins. D, thionin isolated from *Triticum monococcum*. E, thionin isolated for *T. urartu*.](image)

**HYDROLYSIS OF β-PUROTHIONIN WITH CLOSTRIPAIN**

![Fig. 9. High performance liquid chromatography separation of clostripain-hydrolyzed pyridylethylated purothionin peptides for amino acid analysis. A, purothionin reacted with clostripain for 8 min. B, purothionin hydrolyzed with clostripain for 4 hr. Numbers over peaks designate peptide fractions collected for analysis.](image)
Six different amino acids were present in peptide 7, one residue each of aspartic acid (D), from acidic hydrolysis of asparagine (N), alanine (A), arginine (R), valine (V), and leucine (L) and two residues of PECs (PC). From the known sequence of β-purothionin (Fig. 1), the only place such a peptide occurs is from residues 24 through 30. The peptide must have been formed when clostripain hydrolyzed the purothionin adjacent to residues 23 (lysine) and 30 (arginine). The peptide was thus released by a (presumably) fast hydrolysis at arginine and then a much slower cleavage at lysine, since peptide 7 was not present after 8 min of proteolytic hydrolysis (Fig. 9A).

Looking at the amino acid compositions of 10 of the 11 280-nm-absorbing fractions present in the 4-hr hydrolysate sample (fraction 4 was inadvertently discarded), all but one (peptide 3) yielded amino acids on HCl hydrolysis (Table I). "Peptide" 3 apparently contained nonpeptide material that absorbed at 280 nm. The peptide fractions in the 4-hr sample that were also present in the 8-min hydrolysate (fractions 1, 6, 8, 9, and 11) contain amino acids that exactly correspond to peptides covering β-purothionin amino acid residues of 18-19, 1-10, 20-30, 11-17, and 31-45, respectively. These peptides comprise all of the peptides expected if the purothionin were hydrolyzed at each and every arginine residue. All were essentially free of contamination by other peptide material, except fraction 1. Fraction 1 contained all peptides or other materials that did not interact with the column packing; i.e., they eluted in the void volume. After 4 hr of reaction, this fraction contained equal amounts of alanine + arginine and of PECs + lysine. Presumably the PECs-lysine material was not present in the peptide fraction 1 after 8 min of hydrolysis, since it showed no 280-nm-absorbance peak due to the highly absorbing PECs—only the usual void volume disturbance was seen (Fig. 9A). After 4 hr of hydrolysis, however, a distinct PECs-lysine peak was present in the area immediately after, and collected together with, the void volume. From this, it is probable that the very small peptide alanyl-arginine eluted in the void volume after 8-min proteolytic hydrolysis, but that the PECs-lysine was present only in the 4-hr hydrolysate. To summarize, all peptides formed within 8 min of clostripain hydrolysis of β-purothionin arose from cleavages adjacent to and on the C-terminal sides of arginine residues, and all peptide bonds adjacent to arginine residues were completely hydrolyzed.

Analysis of the amino acids in those fractions present in 4-hr hydrolysates but not in 8-min hydrolysates demonstrates that of the extra peptides formed, four (peptides 1, 2, 7, and 10) arose from proteolytic hydrolysates on the C-terminal sides of lysine residues (residues 32, 5, 23, and 32, respectively). None of these hydrolysates occurred adjacent to the lysine residues 1 or 41. N-terminal amino acid residues are often resistant to proteolytic hydrolysis, which may explain why residue 1 was immune to attack. The peptide bond between residues 41 and 42 may have been resistant to enzymatic hydrolysis because residue 42 is a negatively charged asparagine. It is known that the rate of hydrolysis of proteins by trypsin is reduced significantly when lysine residues are N-terminal or are adjacent to amino acid residues whose side chains have a net negative charge (Kasper 1975). The hydrolysis event that released peptide 5 (residues 11-14) occurred at the C-terminal side of asparagine. Neither clostripain nor trypsin normally hydrolyzes proteins at asparagine residues, and in this experiment no sign of any hydrolysis at asparagine residue 11 was found. Something about the structure of purothionin apparently makes the molecule

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**Fig. 10.** Amino acid composition analysis of one peptide (peptide 7) isolated by high performance liquid chromatography from a 4-hr clostripain hydrolysis of pyridylethylated β-purothionin. D = aspartic acid, A = alanine, R = arginine, V = valine, PC = pyridylethyl cysteine, and L = leucine.
### TABLE I
Amino Acid Compositions of Peptides from a 4-Hr Clostricipan Hydrolysis of β-Purothionin*

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<td>Picomoles of peptide</td>
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<td>0</td>
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<td>260</td>
<td>700</td>
<td>640</td>
<td>960</td>
<td>950</td>
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*Residues, rounded off to nearest whole number.
*Average amount of each amino acid in peptide.

especially susceptible to hydrolysis between residues 14 and 15. Peptides corresponding to residues 6–10, 20–23, and 15–17 that would also have been liberated by the hydrolyses at lysine or asparagine residues 5, 23, and 14, respectively, were not analyzed. Neither of the first two of these peptides contained residues that absorbed at 280 nm, so they were not collected. Two small peaks of material that absorbed at 220 nm but not at 280 nm were present but were not analyzed. They eluted just before the collected peak 2 and between peaks 4 and 5 and probably contained residues 6–10 and 20–23. Peptide 4, which absorbed light at 280 nm but was lost before analysis, probably contained the peptide encompassing residues 15–17. From the amounts of peptide materials present in the various 4-hr peptide fractions (Table 1), the hydrolyses at lysine and asparagine residues were about 75% (residue 5), 29% (residue 14), 52% (residue 23), and 76% (residue 32).

It is apparent from these examples that HPLC analyses of peptide fragments of proteins and of their amino acid compositions can yield much useful information about the structures of the original protein substrates. This is especially true when data are obtained from the hydrolysis of the protein with several different proteases of varying specificities. By combining these methods with chemical microsequencing and enzymatic (carboxypeptidase and aminopeptidase) analyses, complete amino acid sequences of small amounts of proteins can be obtained.

### LITERATURE CITED


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