AMINO ACID SEQUENCES OF THE TWO α-PUROTHIONINS OF HEXAPLOID WHEAT[1]

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ABSTRACT   Cereal Chem. 54(3): 511-523

α-Purothionin extracted from flour milled from hexaploid wheat was separated by ion-exchange chromatography into two pure proteins. The two proteins, α1- and α2-purothionins, were present in approximately equal amounts and differed in amino acid composition by eight residues. Native and reduced and pyridylethylated α1- and α2-purothionins were hydrolyzed with chymotrypsin to obtain peptides suitable for amino acid sequence studies. The complete amino acid sequences of the two α-purothionins were determined. The α1- and α2-purothionin molecules each consist of 45 amino acid residues. Their amino acid sequences differ at six positions. Three of the differences are substitutions of chemically similar amino acids. None of the substitutions alters the net charge of the proteins. The α1-purothionin contains isoleucine at position 33, where α2-purothionin has leucine; this accounts for the often-observed fact that α-purothionin as extracted contains only a half residue of isoleucine. The α1- and α2-purothionin sequences differ from that of β-purothionin at 5 and 6 positions, respectively, with one amino acid change in each case (glycine-aspartic acid, residue 42) which alters the net charge on the α-proteins as compared to the β-purothionin. The amino acid sequences of the α-purothionins show considerable homology to those of the viscotoxins of mistletoe, as has already been shown for β-purothionin.

Wheat purothionin is a low-molecular-weight (mol wt), basic protein which can be extracted from wheat flour with ether as a lipoprotein complex (1). It can also be extracted with sodium chloride solution (2–4) or dilute acid (1).

Purothionin differs markedly from other flour proteins. It contains large amounts of cystine (about 20%) and of basic amino acid residues (arginine + lysine about 20%), but no histidine, methionine, or tryptophan, and only small amounts of glutamic and aspartic acids and proline (4,5). On gel electrophoresis, purothionin resolves into two components of similar mobility, designated as α- and β-purothionin (4). The mol wt of each purothionin is about 4900 (6,7). The function of the purothionins in wheat endosperm is not known, but they are toxic to animals when injected intravenously or intraperitoneally (8), and to some bacteria (9,10) and yeasts (9,11,12).

The amino acid sequences of several purothionin preparations have been determined. Ohtani et al. (12) have reported the sequences of two purothionins, A-I and A-II, isolated from a wheat purothionin "A" preparation. We have determined the sequence of β-purothionin (7). Our β-purothionin has the same sequence as the A-1 purothionin of Ohtani et al. (12).

This article reports the amino acid sequences of two distinct components of α-purothionin (designated α1 and α2), obtained from hexaploid wheat, which differ in amino acid composition and sequence but not in electrophoretic mobility. The sequence of α1-purothionin is the same as that of the A-II purothionin of Ohtani et al. (12).

MATERIALS AND METHODS

Preparation of α_1- and α_2-Purothionins

The α_1- and α_2-purothionins were prepared from α-purothionin[3] by ion-exchange chromatography. The α-purothionin was isolated from a petroleum ether extract (6) of flour milled from a sample of Canadian hard red spring wheat *(Triticum aestivum* L., cv. Manitou).

For the separation, the α-purothionin (100 mg) was dissolved in 10 ml of 0.3M ammonium acetate buffer, pH 5.2, and applied to a 2×25-cm column containing CM-cellulose (Whatman CM-52) equilibrated with the same buffer. The sample was eluted with a 1600-ml linear concentration gradient of ammonium acetate, pH 5.2, from 0.4 to 0.7M. The flow rate was 30 ml/hr and the effluent was monitored at 254 and 280 nm.

The α_1- and α_2-purothionins were only partially resolved (Fig. 1) but fractions collected from the distal portions of the absorption peaks yielded pure α_1- and α_2-purothionins as determined by amino acid analysis. The fractions were freeze-dried twice to remove the volatile ammonium acetate.

Preparation of Chymotryptic Peptides and Sequencing

The materials and methodology used to obtain chymotryptic peptides for amino acid sequencing and to sequence the proteins and peptides were the same as those previously used to sequence β-purothionin (7).

C-Terminal Analyses

C-terminal amino acid residues of the α-purothionins were determined using fraction A of the wheat carboxypeptidase preparation of Preston and Kruger

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[3]α-Purothionin is the mixture of α_1- and α_2-purothionins as extracted from flour, before separation into the α_1- and α_2- components.
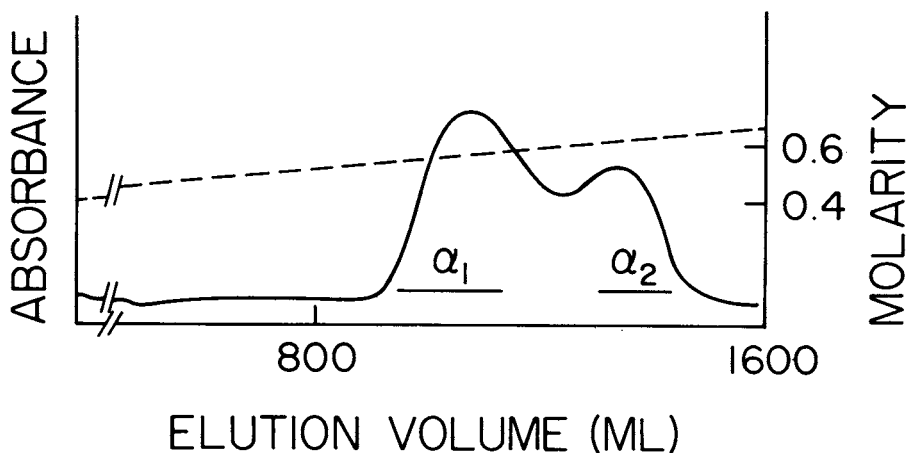

Fig. 1. Separation of α-purothionin into α_1- and α_2-purothionin fractions on carboxymethyl cellulose at pH 5.2; ———— absorbance at 280 nm; - - - molarity of ammonium acetate in gradient.

(13) to sequentially remove the terminal residues. This enzyme has been shown to possess carboxypeptidase-C-type activity (13). Analyses were made as described previously for β-purothionin (7), except that the fraction A enzyme was used instead of fraction B.

RESULTS AND DISCUSSION

Purification of α_1- and α_2-Purothionin

While purifying α- and β-purothionin for sequencing studies by ion-exchange chromatography, it was noted that β-purothionin gave a symmetrical elution peak, whereas the α-counterpart did not. This behavior suggested that the α-purothionin preparation was probably heterogenous. This possibility was strengthened when amino acid composition analysis of chymotryptic peptides of α- and β-purothionins showed that three peptide fractions contained mixtures of two apparently homologous peptides, half of which contained one isoleucine residue while the other half had none (6). These data agreed with the findings of other laboratories (10,14,15) which showed the presence of a half residue of isoleucine in α-purothionin per 5,000 Daltons mol wt.

When our α-purothionin preparation was applied to the CM-cellulose column and eluted with a shallow linear gradient of ammonium acetate, it separated into two components; these will be designated α_1- and α_2-purothionins (Fig. 1). The amounts of α_1- and α_2- components varied somewhat but the two were generally present in approximately equal amounts.

Amino Acid Compositions of α_1- and α_2-Purothionins

Fractions were collected as indicated in Fig. 1 and the individual proteins were isolated, hydrolyzed in 6N HCl, and their amino acid compositions determined (Table I). The amino acid compositions of the two α-purothionins differed by

TABLE I
Amino Acid Compositions of α_1- and α_2-Purothionins[a]

Amino Acid	α_1-Purothionin	α_2-Purothionin
Lysine	5.8	5.6
Histidine	0	0
Arginine	5.6	6.1
Aspartic acid	2.2	2.3
Threonine	1.2	4.0
Serine	4.5	5.2
Glutamic acid	1.2	1.1
Proline	2.3	2.3
Glycine	5.2	4.6
Alanine	3.2	1.2
Half-cystine	8.0	8.6
Valine	1.0	1.0
Methionine	0	0
Isoleucine	0.9	0
Leucine	4.2	5.3
Tyrosine	0.9	0.9
Phenylalanine	1.0	1.0

[a]Based on one phenylalanine residue per mole.

approximately 8 residues. α_1-Purothionin contained one residue of glycine, two of alanine, and one of isoleucine above those in α_2-purothionin. On the other hand, α_2-purothionin had four additional residues, three threonines, and one leucine. These data showed clearly that α_1-purothionin contains one isoleucine residue, while α_2-purothionin contains none. This accounts for the one-half residue found in many α-purothionin preparations. The amino acid compositions of α_1- and α_2-purothionins differ from the β-purothionin composition (7) by 9 and 11 residues, respectively.

Chymotryptic Peptides

When α-, α_1-, and α_2-purothionins were subjected to limited chymotryptic hydrolysis (15 min, native protein) and the resulting peptides were reduced, pyridylethylated, and separated by ion-exchange chromatography on CM-cellulose with an ammonium acetate gradient (6), the peptide patterns shown in Fig. 2 were obtained. The α-purothionin preparation (Fig. 2A) yielded three major peptide-containing peaks: chα14, chα16 and chα17 (using the numbering system of reference 6). The α_1-purothionin (Fig. 2B) yielded only two peptide peaks, ch$\alpha_1$14 and ch$\alpha_1$17, which eluted at the same ammonium acetate concentrations as the two larger peaks obtained from α-purothionin. The α_2-purothionin was cleaved into three peptides (Fig. 2C), which eluted at the same volumes as the three peptides from α-purothionin.

The eluate from each peak in Fig. 2 was collected and freeze-dried to obtain peptides for amino acid composition and C-terminal residue analysis. The ch14 peptides from α-, α_1-, and α_2-purothionins each contained 13 amino acid residues and had C-terminal tyrosine residues as determined with carboxypeptidase (Table II). Since the native proteins all had lysine as C-terminal residues, it seemed probable that the ch14 peptides arose from hydrolysis at the single
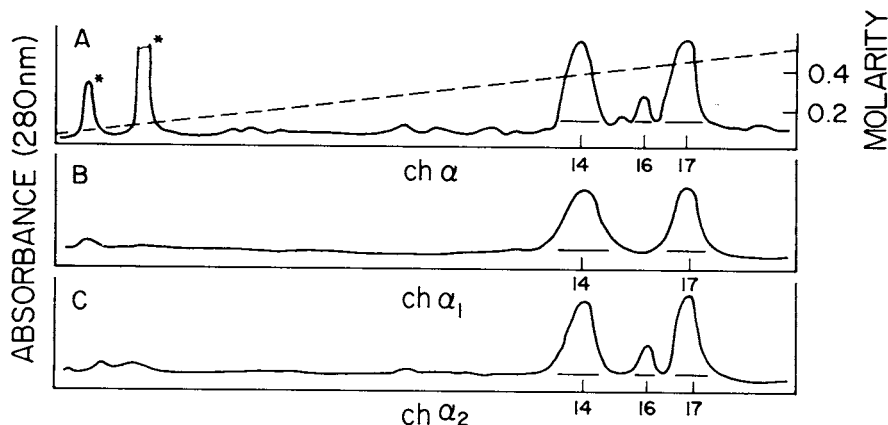

Fig. 2. Elution patterns of peptides from limited chymotryptic hydrolysis of purothionins from a CM-cellulose column. (A) α-purothionin; (B) α_1-purothionin; (C) α_2-purothionin; - - - molarity of ammonium acetate in gradient. The two peaks marked * contained no peptide material.

tyrosine residue of each protein and represented the N-terminal ends of the native proteins. Furthermore, the compositions of these peptides corresponded closely with those of the first 13 residues of β-purothionin (7). The ch$\alpha_1$14 and ch$\alpha_2$14 peptide compositions differed in one residue only; the ch$\alpha_1$14 contained an extra serine residue which apparently replaced a threonine of ch$\alpha_2$14. The sums of the amino acid residues found in peptides chα17, ch$\alpha_1$17, and ch$\alpha_2$17, when added to those of their ch14 counterparts, account for all of the residues of the native protein, indicating that the ch14 and ch17 peptides arise via single hydrolytic scissions of the original proteins. As expected, all of the ch17 peptides had lysine at their C-termini. The ch$\alpha_1$17 and ch$\alpha_2$17 peptides differed in 4 amino acid pairs. Ch$\alpha_1$17 had two additional residues of alanine, one of glycine, and one of isoleucine, while ch$\alpha_2$17 had one additional leucine, one serine, and two threonines.

The ch16 peptide, formed in relatively small amounts in digests of α- and α_2-purothionin preparations, contained 20 residues; carboxypeptidase analysis revealed that both chα16 and ch$\alpha_2$16 had leucine as the C-terminal residue. Since the ch16 peptides contained amino acids which were all also found in the corresponding ch17 peptides, including the single valine and glutamic acid residues of the parent proteins, it was apparent that they were formed from the ch17 peptides by hydrolysis at one specific leucine residue. It has been shown (6)

TABLE II
Amino Acid Compositions and C-Terminal Residues of Limited Hydrolysis Chymotryptic Peptides[a]

Amino Acid	ch14			ch16		ch17		
	α	α_1	α_2	α	α_2	α	α_1	α_2
Lysine	1.0 (1)[b]	0.9 (1)	1.0 (1)	2.0 (2)	2.2 (2)	4.6 (5)	4.6 (5)	4.7 (5)
Arginine	2.0 (2)	2.1 (2)	2.0 (2)	3.2 (3)	3.0 (3)	3.7 (4)	3.6 (4)	3.8 (4)
4-PE-Cys[c]	2.8 (3)	3.2 (3)	2.7 (3)	4.3 (4)	4.4 (4)	4.6 (5)	5.3 (5)	5.4 (5)
Aspartic acid	1.0 (1)	1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Threonine	1.0 (1)	1.0 (1)	1.7 (2)	1.0 (1)	0.8 (1)	1.0 (1)	...	1.9 (2)
Serine	1.8 (2)	1.8 (2)	1.2 (1)	1.6 (2)	2.0 (2)	3.5 (4)	3.1 (3)	4.0 (4)
Glutamic acid	...	...	...	1.1 (1)	1.0 (1)	0.8 (1)	1.0 (1)	1.1 (1)
Proline	...	...	...	...	...	2.1 (2)	2.0 (2)	2.0 (2)
Glycine	1.0 (1)	1.1 (1)	1.0 (1)	1.4 (1)	1.2 (1)	4.2 (4)	4.1 (4)	3.4 (3)
Alanine	...	...	...	1.3 (1)	1.1 (1)	3.0 (3)	3.0 (3)	1.4 (1)
Valine	...	...	...	1.0* (1)	1.0* (1)	1.0* (1)	1.0* (1)	1.0* (1)
Isoleucine	...	...	...	...	...	0.8 (1)	0.9 (1)	...
Leucine	1.0* (1)	1.0* (1)	1.2 (1)	3.1 (3)	3.0 (3)	3.3 (3)	3.0 (3)	4.1 (4)
Tyrosine	0.8 (1)	1.0 (1)	1.0* (1)	...	...	...	...	...
Phenylalanine	...	...	...	...	...	1.0 (1)	1.0 (1)	1.0 (1)
C-Terminal residues	Tyr	Tyr	Tyr	Leu	Leu	Lys	Lys	Lys

[a]Results are mole ratios with respect to the amino acid marked * in each column.
[b]Number in parentheses has been adjusted to the nearest integer.
[c]Pyridylethylated cysteine.

that chymotrypsin cleaved purothionins at positions adjacent to leucine residues, but at a relatively slow rate.

The ch14 and ch17 peptides from α-purothionin have amino acid compositions intermediate between those from the α_1- and α_2-purothionins as expected and the identity of chα16 and ch$\alpha_2$16 shows that the chα16 arose from the α_2-purothionin component of α-purothionin.

Hydrolysis of reduced and pyridylethylated α-, α_1-, and α_2-purothionins for 30 min with chymotrypsin yielded 17 peptides from each protein. When the peptides were separated by CM-cellulose chromatography, the α_1- and α_2-purothionin preparations both yielded elution patterns identical with those already reported for α-purothionin (6), although the amino acid compositions of some of the α_1- and α_2-peptides were slightly different. The only 30-min hydrolysis peptides necessary for sequencing the α_1- and α_2-purothionins were ch$\alpha_1$6 and ch$\alpha_2$6, which had identical amino acid compositions, including both proline residues and the single phenylalanine residue of the parent proteins (Table III).

Partial Sequences of Reduced and Pyridylethylated α_1- and α_2-Purothionins (PEα_1 and PEα_2)

Reduced and pyridylethylated samples (10 mg) of α_1- and α_2-purothionins were each subjected to 18 cycles of automated Edman degradation using the methods reported previously (7). The results are shown in Fig. 3A. The two proteins differed in two residues in this region, with serine 6 of α_1-purothionin replaced by a threonine in α_2-purothionin, and the alanine at position 18 replaced by serine. This corroborated the peptide composition data by showing that the single tyrosine residue of each protein was located at position 13 and that there was a single serine-threonine replacement in the parts of the two proteins corresponding to ch$\alpha_1$14 and ch$\alpha_2$14.

Since it seemed probable that the ch17 peptides started at residue 14 of each purothionin, the sequences of PEα_1 and PEα_2 were only determined through 18 residues, even though preliminary experiments showed that analyses of up to 32 residues could be accomplished.

Partial Sequences of Reduced and Pyridylethylated ch$\alpha_1$17 and ch$\alpha_2$17

When the larger chymotryptic peptides, ch$\alpha_1$17 and ch$\alpha_2$17, were sequenced after being reduced and pyridylethylated, the results shown in Fig. 3B were obtained. The first 5 residues of both peptides had the same amino acid sequences

TABLE III
Amino Acid Compositions of Peptides ch$\alpha_1$6 and ch$\alpha_2$6[a]

Amino Acid	ch$\alpha_1$6	ch$\alpha_2$6
Lysine	1.9 (2)	1.8 (2)
PE-Cys	0.8 (1)	0.9 (1)
Serine	0.9 (1)	0.9 (1)
Proline	1.9 (2)	1.9 (2)
Glycine	1.0 (1)	0.8 (1)
Phenylalanine	1.0 (1)	1.0 (1)
C-Terminal residue	Lysine	Lysine

[a]Results are mole ratios with respect to phenylalanine.

as were found at positions 14 to 18 of PEα_1 and PEα_2, confirming that the ch17 peptides arose from cleavage of the bonds adjacent to residue 13 of each purothionin. Twenty-nine sequence cycles were attempted on each ch17 peptide, but no useful data were obtained after cycle 27 (residue 40 of the original proteins) where proline was detected in both peptides.

Five differences were found in the portions of the ch17 peptides that were sequenced: the residues alanine 5, alanine 13, glycine 14, isoleucine 20, and serine

Fig. 3. Partial amino acid sequences of PEα_1 and PEα_2 and complete or partial sequences of chymotryptic peptides used to determine the complete sequences of the α_1- and α_2-purothionins. (A) Sequences of the first 18 amino acids from the N-terminus of PEα_1 and PEα_2; (B) partial sequences of chymotryptic peptides ch$\alpha_1$17 and ch$\alpha_2$17; (C) complete sequence of chα6, determined after derivation of the peptide with ANS; and (D) sequences of the first four residues from the C-terminus of native α_1- and α_2-purothionins as determined using a germinated wheat seed carboxypeptidase.

21 of chα₁17 were replaced by serine 5 (as seen also with PEα₂), serine 13, threonine 14, leucine 20, and threonine 21 of chα₂17. These replacements account for all of the differences indicated by amino acid compositions of the two peptides (8 amino acid differences). An additional difference in the sequence which does not affect the composition is the deletion of serine from chα₁17 at one position (residue 5 or 13) and from chα₂17 at a different position (residue 21).

Complete Sequence of Reduced and Pyridylethylated chα₁6 and chα₂6 Peptides

Since amino acid analysis data indicated that α₁- and α₂-purothionins each contained about 45 amino acid residues and 38 of these were positioned by sequencing the whole proteins and the ch17 peptides, it appeared that about seven residues remained to be positioned, including a proline, a phenylalanine (the only one in the protein), and a lysine (the C-terminal residue of each protein). In examining the peptides released by prolonged chymotryptic hydrolysis of the α-purothionins, one (chα₁6 and chα₂6) from each protein was found which contained these amino acids. In addition, each contained a second proline residue. Since there were only two proline residues in each of the parent purothionins, it was apparent that one of the ch6 peptide prolines must overlap with the proline residue previously found in the ch17 peptides which occupied position 40 of each native protein.

Since the six amino acid differences found in sequencing the PEα-purothionins and ch17 peptides accounted for all of the changes expected from amino acid composition differences, and because chα₁6 and chα₂6 peptides had identical amino acid compositions, they were not sequenced separately, but as a mixture, chα6. This peptide mixture was treated with 2-amino-1, 5-naphthalene-disulfonic acid (ANS) to make it less soluble in the sequencing solvents (7) and sequenced. The sequence obtained is shown in Fig. 3C. There was no evidence of heterogeneity at any of the positions, confirming the assumption that both components (chα₁6 and chα₂6) of the chα6 mixture have identical sequences.

The C-terminal lysine residue, which reacted with the ANS, could not be detected by the gas-liquid chromatography method used (7) and was positioned by subtraction of the first seven residues sequenced from the amino acid composition of the whole peptides. Carboxypeptidase hydrolysis confirmed that the C-terminal residue was lysine.

The proline residue at position 3 of chα6 must be the same as that previously found at position 27 of the ch17 peptides (position 40 of whole protein), since there are only two proline residues in either α₁- or α₂- purothionin. This residue thus provides evidence that the ch17 and ch6 peptides overlap. The sequencing of chα6 thus allowed the positioning of residues 38 to 45 of the proteins.

Determination of the C-Terminal Sequence

The sequencing of residues 1−45 accounted for all of the amino acids found by amino acid analysis of the native proteins, with the possible exception of one lysine and one arginine residue of each purothionin. The question then arose as to whether the amino acid analysis values for these basic residues were slightly high, or whether there were two additional residues at positions 46 and 47 of the proteins. It did not seem likely that the sequence was -Lys (45)-Arg (46)-Lys (47)-COOH, since carboxypeptidase B enzyme released only one equivalent of lysine from each protein, not the two equivalents of lysine and one of arginine expected

if they contained 47 residues.

To obtain conclusive evidence, native α_1- and α_2-purothionin samples were subjected to hydrolysis by a germinated wheat seed peptidase possessing carboxypeptidase-C-type activity (13). The results obtained when the two purothionins were hydrolyzed with this enzyme are shown in Fig. 4. It is obvious that in both proteins the order of release (and thus the order in the protein, from the C-terminus) of amino acids was lysine-proline-phenylalanine-glycine (Fig. 3D). This evidence confirms that the lysine at residue 45 is indeed the C-terminal residue of both proteins.

Comparison of α_1-, α_2-, and β-Purothionin Sequences

Complete amino acid sequences of the two α-purothionins and that of β-purothionin reported earlier (7) are shown in Fig. 5. The three sequences show differences at eight positions: 5, 6, 18, 26, 27, 33, 34, and 42. Six of the variable positions are adjacent to other variable residues and all three proteins differ from each other at position 27. There are six differences between α_1- and α_2-, five differences between α_1- and β-, and six differences between α_2- and β-purothionins.

GENERAL DISCUSSION

The amino acid sequences of α_1-, α_2-, and β-purothionins showed that about half of the amino acid replacements in the purothionins are replacements of amino acids by similar residues such as lysine/arginine, serine/threonine, or isoleucine/leucine, and only one replacement, aspartic acid (β)/glycine (α_1 and α_2) at residue 42, leads to any alteration of the charge properties of the proteins. This change accounts for the fact that β-purothionin possesses a lower
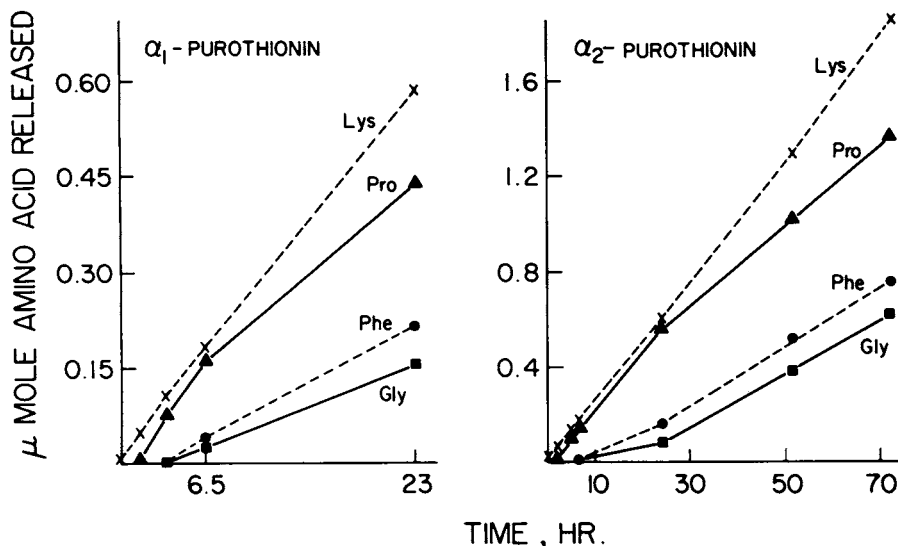

Fig. 4. Release of amino acids from native α_1- and α_2-purothionins with a germinated wheat seed carboxypeptidase.
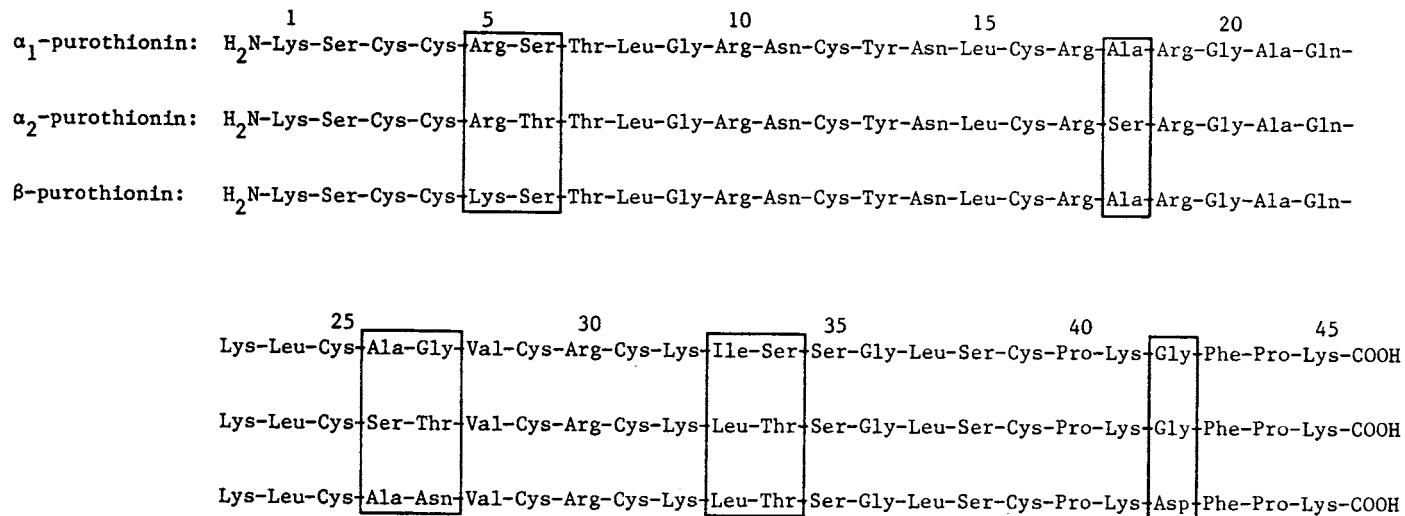
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                           1                    5                   10                  15                  20
α₁-purothionin:  H₂N-Lys-Ser-Cys-Cys-|Arg-Ser|-Thr-Leu-Gly-Arg-Asn-Cys-Tyr-Asn-Leu-Cys-Arg-|Ala|-Arg-Gly-Ala-Gln-

α₂-purothionin:  H₂N-Lys-Ser-Cys-Cys-|Arg-Thr|-Thr-Leu-Gly-Arg-Asn-Cys-Tyr-Asn-Leu-Cys-Arg-|Ser|-Arg-Gly-Ala-Gln-

β-purothionin:   H₂N-Lys-Ser-Cys-Cys-|Lys-Ser|-Thr-Leu-Gly-Arg-Asn-Cys-Tyr-Asn-Leu-Cys-Arg-|Ala|-Arg-Gly-Ala-Gln-


                        25                   30                  35                  40                  45
        Lys-Leu-Cys-|Ala-Gly|-Val-Cys-Arg-Cys-Lys-|Ile-Ser|-Ser-Gly-Leu-Ser-Cys-Pro-Lys-|Gly|-Phe-Pro-Lys-COOH

        Lys-Leu-Cys-|Ser-Thr|-Val-Cys-Arg-Cys-Lys-|Leu-Thr|-Ser-Gly-Leu-Ser-Cys-Pro-Lys-|Gly|-Phe-Pro-Lys-COOH

        Lys-Leu-Cys-|Ala-Asn|-Val-Cys-Arg-Cys-Lys-|Leu-Thr|-Ser-Gly-Leu-Ser-Cys-Pro-Lys-|Asp|-Phe-Pro-Lys-COOH
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Fig. 5. Comparison of the amino acid sequences of α₁-, α₂-, and β-purothionins. Residues which differ in the various proteins are enclosed by boxes.

electrophoretic mobility than the α-purothionins at pH 3.2, and allows the simple separation of α- and β-purothionins by CM-cellulose chromatography. While it is not immediately obvious why the differences between α_1- and α_2- purothionins cause the two proteins to elute differently from CM-cellulose, it may be that the replacement of the aliphatic side-chain amino acids of α_1- purothionin by three residues with hydroxylated side chains may allow more hydrogen bonding between α_2-purothionin and the cellulose of the CM-cellulose column, and thus causes the slight retardation of the α_2-purothionin.

The molecular weights of the three purothionins, calculated on the basis of their amino acid sequences, are α_1-4812, α_2-4916, and β-4913. These values assume that all sulfur-containing amino acids are present as disulfide cystine residues, since all previous work has shown that native purothionins all lack free sulfhydryl groups. These mol wt values are in agreement with those recently determined by Nimmo et al. (5) by sedimentation equilibrium centrifugation, and they are roughly half the values reported by many early workers.

Completion of the primary structures of the purothionins requires the location of the disulfide bridges. Except for residues 3 and 4, the pyridylethylated cysteine (PE-Cysteine) residues are about evenly distributed along the polypeptide chains. Since the PE-cysteine residues occupy identical positions in all three alkylated purothionins, it seems likely that the four disulfide linkages of the native proteins are identical in each case. The disulfide bonds are probably responsible for maintaining the three-dimensional structures of the proteins, but it will be difficult to assign these linkages since that will necessitate specifically hydrolyzing the native protein between residues 3 and 4 and between residues 25 and 29.

The basic residues, arginine and lysine, are evenly distributed along the polypeptide chains and are invariable except for the arginine/lysine replacement at residue 5.

The high degree of homology in the sequences of the three purothionins undoubtedly accounts for the similarity of their secondary and tertiary structures which has been shown by the findings that α- and β-purothionins have identical uv spectra and all possess compactly folded, globular tertiary structures (5). It has been estimated that the secondary structure comprises approximately 40% α- helix (5). The differences in sensitivity of microbes to α- and β-purothionin poisoning found by Fernandez de Caleya et al. (10) must be due to relatively minor differences in structure; possibly to the presence of the negatively charged aspartic acid residue of β-purothionin; especially since this residue is distal to the final cysteine residue of the protein and is relatively accessible, as is shown by the fact that it is readily removed by carboxypeptidase hydrolysis of the native protein.

The chα16 peptide obtained by limited chymotryptic hydrolysis of α_2- purothionin has an amino acid composition which coincides with the sequence for positions 14 to 33 inclusive, indicating it must arise by a hydrolysis of the ch$\alpha_2$17 peptide or the native protein at the bond adjacent to leucine 33. The same hydrolysis cannot readily occur with α_1-purothionin, since its position 33 is occupied by an isoleucine residue and is thus more resistant to chymotryptic hydrolysis. No evidence was ever found for an equivalent peptide from β- purothionin (6), either in short- or long-term chymotryptic digestions. Since the leucine at position 33 of α_2-purothionin is the only one of the five leucine

molecules which is hydrolyzed in 15 min, it may be that position 33 is located in a part of the protein molecule which is especially exposed to the surrounding solution.

Three portions of the molecules (residues 5 and 6, 26 and 27, and 33 and 34) seem to be especially susceptible to amino acid replacement, with two or more changes occurring in adjacent positions, indicating that these regions of the purothionins can accommodate changes without destroying the ability of the proteins to conduct their (as yet unknown) *in vivo* functions. The replacements in two of these variable regions are conservative (5 and 6 and 33 and 34), but the serine/alanine and glycine/threonine/asparagine replacements in positions 26 and 27 are not. The replacements at position 27 are even more remarkable because the replacement of glycine by either threonine or asparagine (or vice versa) requires the alteration of two codon bases (assuming bacterial codons apply in plants). All other substitutions found in the purothionins can result from a single codon base change. Comparison of the sequences of α_1- and α_2-purothionin with that of viscotoxin A_3, a mistletoe toxin, as has already been done with β-purothionin (7), shows that there are 28 of the 46 residues of the viscotoxin which are identical or chemically very similar in all four proteins, suggesting that they have probably evolved from some common ancestor.

Ohtani *et al.* (12) have recently published the sequences of two proteins from wheat which are toxic to yeasts and which they designated purothionins A-I and A-II. Their sequences are identical to the sequences we have determined for β- and α_1-purothionins, respectively. They isolated their purothionins by a method different from the one used in this study. It is interesting that their method yielded a mixture of α_1- and β-purothionins (which differ by one charge unit) but which did not contain the α_2-purothionin which is identical to the α_1-protein in net charge. They report that their purothionin A, from which the A-I and A-II fractions were obtained, contained dimerized material which could explain their findings if α_1- and β-purothionin form a dimeric complex from which α_2-purothionin is excluded. No evidence was found for any purothionin dimers in the present study.

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