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γ-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm

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Two homologous sulfur-rich basic polypeptides form wheat endosperm, so-called γ_1-purothionin and γ_2-purothionin, are described. Purification involves extraction with volatile solvents and ammonium bicarbonate fractionation followed by reversed-phase high-performance liquid chromatography. The complete primary structure of these two polypeptides has been determined by automatic degradation of the intact, S-carboxymethylated γ-purothionins and peptides obtained by enzymatic cleavage. γ_1-Purothionin and γ_2-purothionin consist of 47 amino acids with an assessed molecular weight of 5239 and 5151 Da, respectively and 8 cysteines organized in 4 disulfide bridges. They present a high degree of homology among themselves (89% of identity) and are the first two thionin-like polypeptides, so-called γ-thionins, described from wheat endosperm.

Wheat thionin; Amino acid sequence; γ-Purothionin

1. INTRODUCTION

Thionins comprise a group of sulfur-rich and basic low molecular weight proteins originally described in the endosperm of several Gramineae [1,2]. They are toxic to certain strains of yeasts [3,4], cultured cells [5] and insect larvae [6], and they modify membrane permeability in cultured mammalian cells [7] and inhibit in vitro protein synthesis in cell-free systems derived from wheat germ [8], rabbit reticulocytes, *Artemia* embryos and mouse liver (unpublished results). However, their physiological function is still unknown.

A total of 5 thionins of known sequence α_1-, α_2- and β-purothionin and α- and β-hordothionin from wheat and barley endosperm, respectively, have been reported a long time ago [8–14]. All of them contain 45 amino acid residues, 4 disulfide bridges [8–10] and present among themselves a high degree of homology.

Thionins are also cysteine-rich related polypeptides isolated from different plants which present similar properties and amino acid sequences: the non-toxin crambin from the crucifer *Crambe abyssinica* [15] and the toxins pyrularia from the parasitic plant *Pyrularia pubera* [16] and viscotoxin from European mistletoe [17]. They consist of 46 amino acids and 3 disulfide bridges and present strong homology with the wheat and barley thionins [15–17].

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Abbreviations: γ_1-P and γ_2-P, γ_1- and γ_2-purothionins; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ch, chymotrypsin

In this paper we report the characterization of two new homologous thionins from wheat endosperm, which, in accordance with their amino acid sequences, seem to belong to a new type of thionins, different to the α- and β-thionins, which we have tentatively called γ-thionins.

2. MATERIALS AND METHODS

2.1. *Extraction and purification*

Salt (NaCl)-soluble proteins from a chloroform/methanol extract of wheat endosperm (*Triticum turgidum* L. cv. Senatore Capelli) was obtained as in [18], followed by extraction with ammonium bicarbonate as also described in [19]. The bicarbonate thionin fraction was first chromatographed by a RP-HPLC system with a Nucleosil C_4 silica column (6 × 250 mm) and eluted with a gradient of acetonitrile containing 0.1% trifluoroacetic acid. Finally, the thionins were repurified by the same elution system but with a shallower gradient. Mini-slab SDS-PAGE and amino acid analyses were performed as previously described [19].

2.2. *Enzymatic cleavage and peptide fractionation*

Native γ-purothionins were reduced and S-carboxymethylated as reported in [19]. The S-carboxymethylated proteins were digested with chymotrypsin (EC 3.421.1) in 0.2 M N-methylmorpholine (pH 8.5) at a protein/enzyme ratio 100:1 (w/w) for 4 h at 37°C. Peptides were fractionated by RP-HPLC on a Novapack C_{18} (3.9 × 150 mm) column. The peptides were eluted with an acetonitrile gradient containing 0.1% trifluoroacetic acid.

2.3. *Sequence determination*

The native, S-carboxymethylated γ-purothionins and chymotryptic peptides were sequenced as reported [19] in a Knauer Modular Liquid Phase Protein Sequencer Model 810 equipped on line with a Knauer PTH-amino acid analyzer.

3. RESULTS AND DISCUSSION

The fractionation by RP-HPLC of the purothionin fraction obtained from the chloroform/methanol ex-

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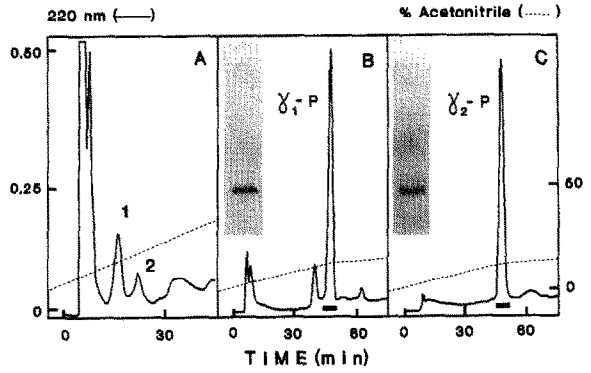

Fig. 1. (A) Fractionation by RP-HPLC of the bicarbonate thionin extract from wheat endosperm. The column was eluted at room temperature with acetonitrile gradients from 10 to 35% containing 0.1% trifluoroacetic acid. Flow rate 1.0 ml/min. (B) and (C): Rechromatography of peaks 1 and 2 in the same column with a shallower gradient of acetonitrile. SDS-PAGE of fractions indicated by bars are inserted.

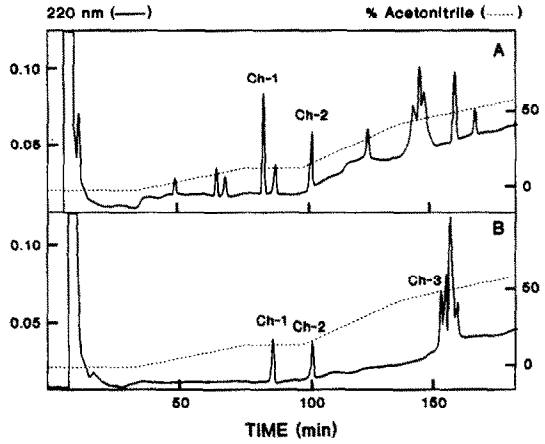

Fig. 2. Fractionation by RP-HPLC of chymotryptic peptides from the S-carboxymethylated γ_1-P (A) and γ_2-P (B). Peptides were eluted at room temperature with acetonitrile gradients from 0 to 50% containing 0.1% trifluoroacetic acid. Flow rate: 0.5 ml/min.

tract of wheat endosperm by extraction with NaCl and precipitation with ammonium bicarbonate is shown in Fig. 1A. Peaks 1 and 2 showed main components of about 5 kDa contaminated by other proteins present in low amounts (data not shown). Contaminants were eliminated by rechromatography in the same RP-HPLC column (Fig. 1B, C). The homogeneity of the rechromatographed protein fractions 1 and 2 was analyzed by SDS-PAGE (see gels inserted in Fig. 1B, C). The purified components were called γ_1-P and γ_2-P, respectively.

The amino acid compositions of both components contain no histidine, tyrosine or threonine, but are rich in cysteine and basic amino acids (data not shown).

The amino acid sequences of γ_1-P and γ_2-P were determined by automatic degradation of the intact molecules and peptides obtained by enzymatic cleavage with chymotrypsin. The RP-HPLC of the chymotryptic digest of the S-carboxymethylated γ_1-P and γ_2-P are shown in Fig. 2A, B.

The analysis of the N-terminal sequence of the native γ_1-P was determined as far as 46 residues (Fig. 3). Gaps at positions 3, 13, 20, 24, 34, 41, 43 and 47 were confirmed as cysteines and gaps at positions 39, 40 and 45 as arginines by the sequence of the first 24 amino acids of the S-carboxymethylated γ_1-P and by the two chymotryptic peptides Ch-1 and Ch-2 from Fig. 2A.

The summary of the sequence study on the γ_1-P described above is shown in Fig. 3. An identical strategy applied for the sequence determination of γ_2-P by the automatic degradation of native, S-carboxymethylated γ_2-P and the chymotryptic peptides Ch-1, Ch-2 and Ch-3 from Fig. 2B is shown also in Fig. 3.

The two polypeptides γ_1-P and γ_2-P are almost identical and consist of 47 amino acids with a calculated molecular weight of 5239 and 5151 Da, respectively. They present 8 cysteine residues and a high content of basic amino acids (4 lysines and 5–6 arginines). No free sulphydryl groups could be found in either γ_1-P or γ_2-P by titration with 4-vinylpyridine in the absence of dithiothreitol. However, when the two polypeptides were reduced with dithiothreitol, the generated SH groups could be detected by titration with 4-vinylpyridine (data not shown), suggesting the presence of 4 disulfide bridges in γ_1-P and γ_2-P.

The amino acid sequences of wheat, barley and related plant thionins according to their relative degree of homology are shown in Fig. 4 and compared with γ_1-P and γ_2-P. The first group of homologous polypeptides includes the toxins crambin and pyrularia and the hydrophobic viscotoxin. All are 46–47 residues long, exhibit a high degree of homology among them and present 3 disulfide bridges located at positions 3–40, 4–32 and 16–26. The second group shows different genetic variants of thionins from wheat (α_1, α_2 and β) and from barley (α and β). They are polypeptides of 45 amino acids with a high degree of homology (93% of identity) and contain 4 disulfide bridges. These two groups present 45% of identity and share 3 disulfide bridges (Fig. 4).

The amino acid sequences of the two γ-thionins show that they are clearly thionins as demonstrated by their close resemblance to the earlier described thionins, but they present several peculiar structural characteristics distinguishing them from the α- and β-thionins from wheat and barley.

In γ-thionins most of the basic amino acids are placed at both ends, while in the α- and β-thionins they are distributed along the polypeptide chain.

Furthermore, γ-thionins have lysine and cysteine as the N-terminal and C-terminal residues respectively, but in α- and β-thionin at both terminals the residues are lysines.

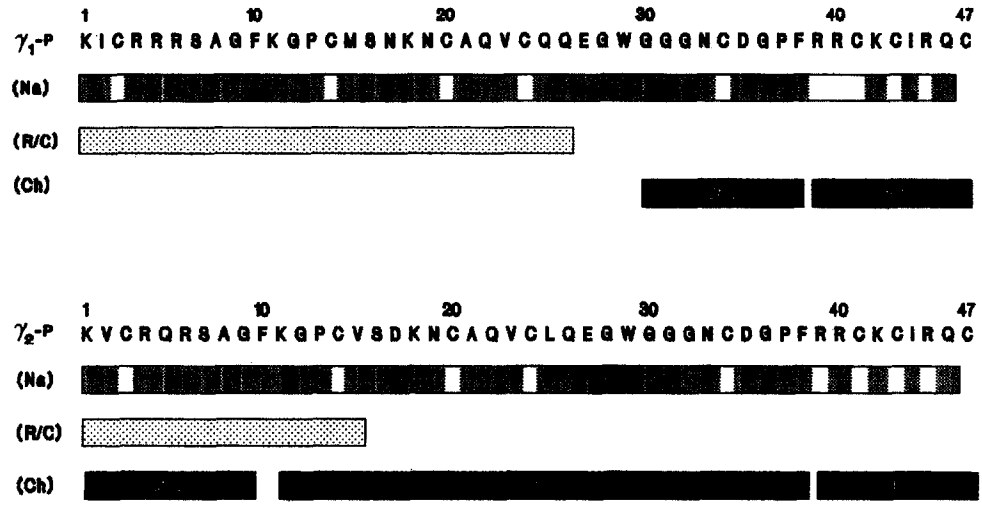

Fig. 3. The alignment of the peptides used for determining the amino acid sequences of the wheat γ_1-purothionin and γ_2-purothionin. Results from automatic sequencing of (Na) the native, (R/C) the reduced and S-carboxymethylated γ-purothionins and (Ch) chymotryptic peptides from the S-carboxymethylated γ-purothionins. Open boxes indicate unidentified residues.

Although the organization of the disulfide bridges of γ-thionins remains to be investigated, the cysteines at positions 3, 14, 20 and 34 could probably form two bridges as do the cysteines 3, 16, 25 and 39 from the first and second groups (Fig. 4). On the other hand, the organization of the other two bridges in γ_1-P and γ_2-P must be different since the other 4 cysteines, including the one at the C-terminal end, occupy positions which are not homologous to the α- and β-thionins. Consequently, γ-thionins have only 32% of identity with the α- and β-thionins in comparison with the 89% of identity they have among themselves.

For the above reasons, the described γ-thionins must correspond to a new type of thionins different from the α- and β-thionins previously reported in wheat and barley. Thus, we have given the name of γ-thionins (γ_1-purothionin and γ_2-purothionin) to these two new polypeptides from wheat endosperm.

The existence of these types of polypeptides in other famlies of Gramineae remains to be investigated. In

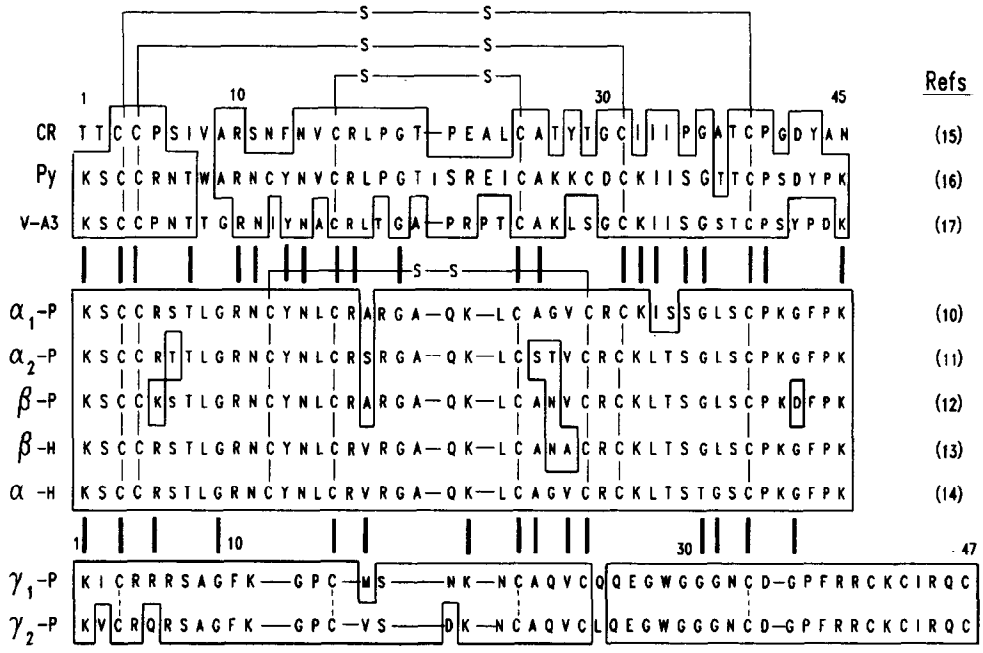

Fig. 4. Amino acid sequence homology between the two wheat γ-purothionins and α- and β-homologous wheat purothionins and barley hordothionins. CR, crambin; Py, pyrularia and V-A3, viscotoxin. Common sequences are indicated in boxes or by vertical bars. Gaps are included to achieve maximal homology.

fact we have recently isolated a new γ-thionin from barley endosperm which presents a 90% identity with γ_1-P and γ_2-P (unpublished results).

The toxic effect of these γ-thionins to certain strains of yeasts, high organisms and cultured cells, as well as the possibility to form immunotoxins specifically toxic to target cells of the conjugated antibody is currently under investigation.

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