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STUDIES OF THE PRIMARY STRUCTURES OF PUROTHIONINS AND PUROTHIONIN-LIKE PROTEINS FROM CEREALS

by

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TO
MY
WIFE
NANETTE

ABSTRACT

Purothionins have been extracted from hexaploid wheat (Triticum aestivum L. cv. Manitou) flour with petroleum ether. The petroleum ether extract was treated with 8% lactic acid and desalted through a Sephadex G25 column. The desalted purothionin preparation was separated on a CM-cellulose (Whatman CM52) column into two fractions, α- and β-purothionins. The α fraction was later separated into subfractions α_1 and α_2. Two modes of chymotryptic hydrolysis were performed on these proteins. Fifteen-min hydrolysis on the native protein yielded 2 major peptides resulting from cleavage at the single Tyr residue. When the protein was reduced and pyridylethylated and then subjected to chymotryptic hydrolysis for 30 min, 15 to 17 peptides were obtained. The amino acid compositions and C-terminal residues of these peptides were analysed and sequence analyses were performed on selected chymotryptic peptides using an Automatic Sequencer (Beckman 890C). Complete sequences of the α_1-, α_2- and β-purothionins were established.

Purothionins were also prepared similarly from the tetraploid species (Triticum durum) and diploid species (Triticum monococcum). Triticum durum possesses 2 purothionins, Duα and Duβ, with amino acid compositions identical to the α_1- and β-purothionins respectively. Triticum monococcum contains 2 purothionins, Tm_a and Tm_b, having identical amino acid compositions and yielding homologous peptides on cleavage with chymotrypsin. The complete sequence of Tm_b was determined using the same procedure used to sequence the β-purothionin. Tm_b and β-purothionin have

identical sequences.

Hordothionins Ho_{a1}, Ho_{a2} and Ho_b were obtained similarly from barley flour. Ho_{a1} and Ho_{a2} have very similar amino acid compositions and charge properties. An unseparated mixture of the 2 forms was sequenced, and heterogeneity was found at 2 residues. The complete sequence of Ho_b was established except for 2 residues.

The primary structures of the purothionins and hordothionins are highly homologous. Not more than 6 changes occur between any two of them and all replacements are between chemically similar residues except one where a Gly in α_1, α_2, Ho_a and Ho_b is replaced by an Asp in β and Tm_b. Most of the replacements require a single base change in the codons specifying the altered amino acids. All these homologous proteins contain 47 residues. Molecular weights obtained on the basis of the sequence data were : α_1 5,096, α_2 5,200, β and Tm_b 5,197, Ho_b 5,126.

The sequence of the first four residues (H_2N-Lys-Ser-Cys-Cys-) of these proteins is totally conserved and is identical to that of the viscotoxins, the purothionin-like toxins from European mistletoe, Viscum album L.. Apparently, the function of these proteins requires the conservation of this portion of the molecule.

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a.a.	amino acid. All amino acids are designated by three-letter symbols. For example, Arg = arginine, Leu = leucine.
ANS	2-amino-1,5-naphthalene-disulfonic acid.
BSA	N,O-Bis(trimethylsilyl)-acetamide.
CEα	carboxyethylated α-purothionin.
CM-cellulose	carboxymethyl cellulose.
C-terminus	carboxyl terminus of protein or peptide.
DFP	diisopropylphosphofluoridate.
DMAA	dimethylallylamine.
EDC	N-ethyl,N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride.
GLC	gas liquid chromatography.
M	molar.
MW	molecular weight.
NBS	N-bromosuccinimide.
N-terminus	amino-terminus of protein or peptide.
PECys	s-β-(4-pyridylethyl)cysteine.
PG	purothionin-like globulins.
PITC	phenylisothiocyanate.
PQ test	phenanthrenequinone test (for PTH-Arg).
PTC	phenylthiocarbamyl.
PTH	phenylthiohydantoin.
Quadrol	N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine.
SDS	sodium dodecyl sulfate.
SPITC	4-sulfophenylisothiocyanate.

TLC	thin layer chromatography.
TPCK	L-1-chloro-3-tosylamido-4-phenyl-2-butanone.
UV	ultraviolet.
V_o	void volume.
V_t	total bed volume.

INTRODUCTION

The purothionins are a group of homologous, low-molecular-weight proteins which can be extracted from wheat flour with organic solvents (Balls et al., 1942a; Nimmo et al., 1968). They are found in hexaploid, tetraploid and diploid species of the Triticum and Aegilops genera (Carbonero and Garcia-Olmedo, 1969). Similar proteins called hordothionins (Redman and Fisher, 1969), are found in barley. More is known about the chemical and physical properties of these proteins than is known about any other flour proteins, but there is still confusion about their molecular weights, genetic origins and functions.

Purothionins and hordothionins have some interesting properties which are quite different from those of other flour proteins. They contain about 20% Cys-Cys, 10% Arg and 10% Lys, but lack Met, Trp and His (Nimmo et al., 1974; Redman and Fisher, 1968). Molecular weights between 5,000 and 12,000 have been reported for these proteins. Recent studies (Nimmo et al., 1974) indicate that purothionins possess about 40% α-helical structure and probably assume a compactly folded, globular shape in solution. Experiments have demonstrated that purothionins are toxic to bacteria (de Caleya et al., 1972), yeasts (Hernandez-Lucas et al., 1974) and small animals when injected either intraperitoneally or intravenously (Coulson et al., 1942). However, their functions in the cereal endosperm are not known. Similar polypeptide toxins, called viscotoxins (Samuelsson, 1961), are found in stems and leaves of European mistletoe Viscum album L.. It has been suggested (de Caleya et al., 1972) that the mode of action of the purothionins is similar to that of small, cyclic polypeptide toxins (eg. gramicidin). Electrophoretic studies on purothionins extracted from various

Triticum and Aegilops species (Carbonero and Garcia-Olmedo, 1969) has led to the postulation that each of the three genomes plays some part in determining the composition of the mixture of purothionins found in hexaploid bread wheat.

This sequence study was undertaken to obtain evidence which would explain the physical and chemical properties of the purothionins and hordothionins. In addition, it was hoped that such knowledge would shed light on the genetic origins of these proteins and would allow speculation about their functions in the endosperm.

LITERATURE REVIEW

PUROTHIONINS AND PUROTHIONIN-LIKE PROTEINS

Purothionins, low-molecular-weight and basic proteins, are extractable from wheat flour by organic solvents. The name was derived from the Greek (πυρος wheat, θειου sulfur) because of the source and the high sulfur content of these proteins (Balls et al., 1942b). Purothionins were first obtained in crystalline form from a petroleum ether extract of bread wheat (Triticum aestivum L.) flour by Balls et al. (1942a). After the initial work, these proteins were not investigated further until the late sixties when several independent studies (Nimmo et al., 1968; Redman and Fisher, 1968, 1969; Garcia-Olmedo et al., 1968) were carried out. The functions, structures and genetic origins of the proteins are not fully known.

Lipopurothionins

Since purothionins are extractable by organic solvents such as petroleum ether, they are probably bound to lipids in flour forming lipoprotein complexes (Balls et al., 1942a; Nimmo et al., 1968). It is not known whether they exist as lipoproteins in the endosperm also. Recently, Hernandez-Lucas et al.* obtained evidence that lipopurothionins

* C. Hernandez-Lucas, R. Fernandez de Caleya, P. Carbonero and F. Garcia-Olmedo. Universidad Politecnica de Madrid, E.T.S. Ingenieros Agronomos, Madrid. Unpublished results.

might be extraction artifacts, the yield of which is determined by the synthesis of the lipid component(s) associated with the proteins.

The nature of the lipid moiety of the lipopurothionin has been investigated by several workers. Balls et al. (1942a) suggested that the purothionins are probably associated with lecithin-like lipoids although only evidence of the presence of phosphorus in the crude product was given. Redman and Fisher (1968) separated lipopurothionins on a Sephadex LH 20 column and found phosphatidyl choline, phosphatidyl ethanolamine, two glycolipids, amino-phospholipids and steroid ester in their fractionated products. Similar findings were reported by Hosney et al. (1970), who identified the two glycolipids as mono- and digalactosyl diglycerides. However, it was not clear whether these lipids were bound to the purothionins or were free lipids which coeluted with the lipopurothionins. Fisher (1970) improved the separation of the lipopurothionins from the contaminating lipids on cross-linked polystyrene beads (Bio Beads $S\text{-}X_2$).

Exposure of lipopurothionins to acids (pH 3) breaks the linkage between the purothionins and the lipid components (Nimmo et al., 1968), suggesting that non-covalent, possibly ionic bonding is involved in the linkage. Hydrochloric acid (Balls et al., 1942a), 8% lactic acid (Redman and Fisher, 1968), and silicic acid (Fisher, 1970) have been used as dissociating agents.

Purification and Fractionation of Purothionins

The lipid-free purothionin preparations are usually contaminated by albumin-like proteins. Further purification has been achieved

with gel electrophoresis, gel filtration, and ion-exchange chromatography. Two fractions, A and B, have been separated using Sephadex G75 (Redman and Fisher, 1968). Fraction A contained mainly albumins; whereas fraction B, containing purothionins, gave two closely spaced bands on starch gel electrophoresis using the aluminum lactate buffer system (pH 3). These bands showed the greatest electrophoretic mobilities of all flour proteins extractable by salt solution. The faster moving (more basic) component was named α-purothionin and the slower was called β-purothionin. Nimmo et al. (1968) separated the crude purothionin extract into three to five fractions on a Sephadex G50 column. Only the fraction containing the lowest molecular-weight-proteins gave the characteristic fast moving doublet on polyacrylamide gel electrophoresis. Redman and Fisher (1968) then quantitatively separated the α- and β-purothionins using ion exchange chromatography (carboxymethyl cellulose). Yields of 45 mg of α-purothionin and 25 mg of β-purothionin were obtained from 2700 g of flour (Redman and Fisher, 1968). Recently, Hernandez-Lucas et al.* extracted purothionins with dilute sulfuric acid and obtained ten times as much protein as was extracted by petroleum ether.

Purothionin-like Globulins

Two globulin proteins from wheat flour have both chemical and physical properties very similar to those of purothionins (Nimmo et al., 1968). These purothionin-like globulins (PG) have the same electrophoretic mobility as the purothionins, appearing as a fast moving doublet

* Unpublished results.

on gel electrophoresis (pH 3). Even at pH 8.5, these proteins move slightly towards the cathode because of their highly basic nature.

The precursors of the PG doublet are soluble in salt solutions (1M NaCl) but not in water. The PG doublet is not seen in the gel electrophoretic pattern at pH 8.5 if the crude extract has not been exposed to low pH during the preparation procedure (Nimmo et al., 1968). The authors suggest that the PG are still linked to lipids before acidification and therefore insoluble in water but soluble in salt solutions. As with lipopurothionin, the linkage is broken once the preparation is exposed to low pH, freeing the water soluble PG doublet.

The PG preparation is similar, if not identical, to purothionins in many other aspects. They both have similar amino acid compositions (Table 1), gel-filtration properties and ultraviolet (UV) spectra (Nimmo et al., 1968). Redman and Fisher (1968) separated the PG into 2 fractions, α and β, having amino acid compositions similar to the corresponding α- and β-purothionins. The α-purothionin and α-PG yielded identical peptide maps on tryptic hydrolyses and had identical C-terminals (Lys).

Amino Acid Compositions

Amino acid compositions of the purothionins and the PG have been analysed by different investigators (Redman and Fisher, 1968, 1969; Nimmo et al., 1968, 1974) and the results are shown in Table 1. For comparision, the amino acid data in Table 1 has been recalculated based on the original reports. The results are expressed in moles of

TABLE 1

AMINO ACID COMPOSITION OF PUROTHIONIN, PUROTHIONIN-LIKE GLOBULIN (PG), AND HORDOTHIONIN.

The results are expressed in mole of amino acid/5,200 g residues recovered, calculated from original papers.

Amino Acid	α-Purothionin		β-Purothionin		α-PG	β-PG	α-Hordothionin
	A	B	A	B	C	C	B
Arg	5.45	5.51	4.52	4.63	5.00	4.03	5.48
Lys	5.50	5.42	6.10	5.72	4.42	5.32	5.55
His	Trace	——	0.19	Trace	0.34	0.09	——
Asp	2.24	2.20	3.53	3.53	1.87	3.36	2.50
Thr	2.96	2.71	2.41	2.39	2.41	1.86	2.86
Ser	5.27	5.25	4.58	4.49	4.21	3.76	4.81
Glu	1.15	1.25	1.36	1.71	1.36	1.23	1.25
Pro	2.18	2.18	2.19	2.41	1.52	1.93	2.32
Gly	4.78	4.71	3.80	3.95	3.74	2.72	4.40
Ala	2.11	2.27	2.96	3.00	1.73	2.50	2.56
½Cys	7.85	8.15	7.87	7.65	6.60	6.50	8.18
Val	1.08	0.93	1.03	1.04	0.81	0.76	1.64
Met	Trace	——	Trace	——	——	——	——
Ile	0.43	0.53	0.33	0.28	0.32	0.15	——
Leu	4.93	4.88	4.90	5.08	4.47	4.47	4.85
Tyr	1.00	0.88	0.99	0.94	0.67	0.75	0.83
Phe	1.10	1.20	1.11	1.23	1.22	1.14	1.12

A: Nimmo et al., 1974.
B: Redman and Fisher, 1969.
C: Redman and Fisher, 1968.

amino acid per 5,200 , which is probably the most reliable reported molecular weight for the purothionins (Nimmo et al., 1974). Purothionins and PGs lack Met, Trp, and His. They contain high percentages of Cys-Cys (≃ 20%), Lys (≃ 10%) and Arg (≃ 10%) but small amounts of Glu (≃ 2%). These proteins are quite distinct from other wheat endosperm proteins which usually contain small amounts of Cys-Cys (≃ 2%), Lys and Arg (≃ 4%), but high percentages of Glu (≃ 30%). There is always about 0.5 residue of Ile per 5,200 MW in the α component while only traces or none are found in the β counterpart. This and genetic evidence (Carbonero and Garcia-Olmedo, 1969) have been interpreted as indications of heterogeneity within the α-purothionin preparation (Nimmo et al., 1974).

Purothionins do not contain free sulfhydryl groups despite their high sulfur contents. All the sulfhydryl groups are involved in disulfide bridges in the native protein (Redman and Elton, 1969; Nimmo et al., 1974). Reduced and alkylated purothionins do not give additional bands on gel electrophoresis. This finding, together with end-group determinations, indicates that purothionins are single-chain polypeptides (Redman and Elton, 1969). All the disulfide bonds in purothionins are fully reduced (> 97%) in 1.5 hr at room temperature in 3M urea using mercaptoethanol. Reduced purothionins in dilute solution (< 30 mg/ml) regain the native conformation after reoxidation, indicating a preferred pairing of the sulfhydryl groups (Redman and Elton, 1969).

Molecular Weight of Purothionins

There has been considerable disagreement about the molecular weights of the purothionins with the older literature reporting molecular

weights of 10,000 to 12,000 (Balls et al., 1942a; Redman and Fisher, 1969), while recent physical measurements suggest a value about half this large (Nimmo et al., 1974). Balls et al. (1942a) reported a minimum molecular weight of 6,000 assuming there was one Tyr and one Phe residue per protein molecule. However, a value of 12,500 was obtained if the ratio of Tyr:Phe:Ile was taken to be 2:2:1 (Redman and Fisher, 1969). Most recently, the molecular weights determined from sedimentation equilibrium measurements were 5,100 and 5,300 for α- and β-purothionins respectively (Nimmo et al., 1974). Part of the confusion about the molecular weights of the purothionins has come from the fact that α-purothionin contains approximately one residue of Ile per 10,000 MW.. This has been ascribed to heterogeneity of the α-purothionin preparation (Nimmo et al., 1974). It appears that the α-purothionin preparation contains a mixture of two components, one of which contains one residue of Ile while the other does not.

Physical Properties of Purothionins

Some physical properties of the purothionins have been characterized by Nimmo et al. (1974). They suggest purothionins have compactly folded, globular structures based on an intrinsic viscosity of 2.7 cc/g. Both α-and β-purothionins have identical circular dichronism spectra indicating 40% α-helical structure. The UV absorption spectra, characteristic of proteins with no or very low tryptophan, show a maximum specific absorbance ($E^{1\%}_{1cm}$) at 278 nm. Emission due to Trp is absent in the fluoresence spectrum and the Tyr emission is strongly quenched,

possibly from interaction with disulfide in the protein. On moving boundary electrophoresis, the purothionin mixture appears as a single peak and has mobilities of 11 and 7.2 x 10^5 cm^2/volt/sec at pHs 3.1 and 8.6 respectively.

Genetic Origins of Purothionins

Carbonero and Garcia-Olmedo (1969) have studied some of the phylogenetic implications of purothionins by investigating the occurrence of α- and β-purothionins in different Aegilops and Triticum species. Both the α and β forms are found in 40 investigated varieties of the allohexaploid Triticum aestivum (genome: AABBDD) and 26 varieties of the allotetraploid Triticum durum (genome: AABB). The β form is found in the diploid species, Triticum monococcum (genome: AA). In contrast, Aegilops speltoides (genome: SS)* and Aegilops squarrosa (genome: DD) both contain the α form. These observations indicate that α- and β-purothionins are the result of divergent evolution at the diploid level and have come to coexist by the process of alloploid formation. If genes in both BB and DD genomes are responsible for the synthesis of α-purothionin, heterogeneity in the α-form is expected in the allohexaploid Triticum aestivum (bread wheat), which possesses both the BB and DD genomes.

Functions of Purothionins

The function of purothionins in the wheat kernel is not known. However, their toxicities towards microorganisms (de Caleya, 1972;

* Genome SS is similar to genome BB.

Hernandez-Lucas, 1974) and small animals (Stuart and Harris, 1942) have been demonstrated. The crystallized purothionin hydrochloride shows both bactericidal and bacteriostatic activities against various strains of bacteria (Stuart and Harris, 1942). A dosage ranging from 1 μg to 50 μg per ml of growth medium is sufficient to inhibit growth of gram-positive organisms but only slightly affects gram-negative organisms. In vivo effects were not found when the anti-bacterial activity of these proteins was tested in mice against pneumococci and streptococci. Anti-bacterial activities were confirmed by de Caleya et al. (1972), who showed that several phytopathogenic bacteria were also sensitive to purothionins. Furthermore, the α and β forms showed different antimicrobial specificities, probably due to differences in the primary structures of these proteins. Fungicidal activity of purothionins toward yeasts, even in high dilution (5 μg/ml culture medium), was first shown by Stuart and Harris in 1942. They suggested that purothionins might be responsible for the yeast-killing property of wheat flour, which was reported as early as 1895 by Jago and was repeatedly cited thereafter (Nose and Ichikawa, 1968; Okada et al., 1970). Recently, Hernandez-Lucas et al. (1974) found that various brewer's yeasts were also affected by the crude purothionin preparations. Purified preparations were less effective than the crude extract. This might be due to other active components present in the crude extract and/or denaturation during preparation of the purified protein.

Purothionins are toxic to small animals when injected either intraperitoneally or intravenously; however, when administered orally, relatively large doses are harmless (Coulson et al., 1942).

Immediately after injection, the animals usually show labored respiration, followed by loss of equilibrium. This is succeeded by a coma, after which death or recovery occurs. A minimum lethal dosage for mice is about 0.3 mg/20 g of body weight, whereas a dose of 2.2 mg to 4.6 mg per kg of body weight is fatal to guinea pigs or rabbits.

Purothionin Analogues from Barley Flour

Analogues of α and β wheat purothionins have been obtained from petroleum-ether extracts of barley flour by Redman and Fisher (1969). These barley proteins were named hordothionin,which was derived from a Latin root ('hordein' is the name commonly applied to barley proteins). Yields of 550 mg of the α form and 35 mg of the β form were obtained from 10 kg of barley flour.

Alpha-hordothionin and α-purothionin have very similar chemical and physical properties. Like α-purothionin, α-hordothionin lacks Trp, Met, His and free sulfhydryl groups (Table 1). Interestingly, Ile is absent from α-hordothionin. The similarity in amino acid compositions (Table 1), tryptic and chymotryptic peptide maps and C-terminal residues between α-hordothionin and α-purothionin suggests they are probably similar in amino acid sequence. Results of immuno-diffusion experiments indicate that α-hordothionin and the purothionins possess common antigenic sites.

Hordothionin-like globulins have been extracted from barley flour with saline solution (Redman and Fisher, 1969). The relationship between these globulins and hordothionins appears to be similar to that between purothionins and purothionin-like globulins.