Identification and Purification of a Purothionin Homologue from Rye (*Secale cereale* L.)

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Purothionins are high sulfur, basic polypeptides present in wheat endosperm, that can be extracted with petroleum–ether (in the form of lipid–protein complexes), and with dilute sulfuric acid. The extraction and purification of a purothionin homologue from rye kernels (*Secale cereale* L.) is reported here for the first time; its close similarity to the β-purothionin from wheat is demonstrated by its identical mobility in one- and two-dimensional electrophoresis and by its amino acid composition.

Purothionins are high-sulfur, basic polypeptides present in wheat flour that were first described by Balls and coworkers (Balls and Hale, 1940; Balls et al., 1942a). They are toxic to higher animals when injected intraperitoneally or intravenously (Coulson et al., 1942), have antibiotic properties against bacteria and yeast (Stuart and Harris, 1942; Fernández de Caleya et al., 1972; Hernández-Lucas et al., 1974), and inhibit papain but not trypsin (Balls et al., 1942b).

In hexaploid wheat (*Triticum aestivum* L.), purothionins are extracted with petroleum–ether in the form of protein–lipid complexes that are readily dissociated by acid treatment (Balls et al., 1942a) and consist of a mixture of two electrophoretic variants, designated α- and β-purothionins (Nimmo et al., 1968; Redman and Fisher, 1968; García-Olmedo et al., 1968; Nimmo et al., 1974). Furthermore, the α class is a mixture of two genetic variants α_B and α_D (Fernández de Caleya et al., 1976). Recently, the amino acid sequences have been established (Ohtani et al., 1975; Mak and Jones, 1976a,b) and it has been found that they closely resemble the viscotoxins isolated from the European mistletoe (*Viscum album* L.) (Samuelsson et al., 1968; Samuelsson and Petersson, 1971).

The ether extraction procedure has yielded purothionin homologues in barley (Redman and Fisher, 1969) and in 22 species of the *Aegilops–Triticum* group (Carbonero and García-Olmedo, 1969). Redman and Fisher (1969) reported that rye (*Secale cereale* L.) lacked a purothionin homologue, and previous repeated efforts from our laboratory (unpublished) also failed to demonstrate the presence of such a molecule.

We have recently reported biochemical and genetic evidence which suggests that the purothionin–lipid complexes might be extraction artifacts, and we have shown also that petroleum–ether soluble complexes can be reconstituted. Furthermore, the yield of petroleum–ether soluble purothionins of tetraploid wheat (*Triticum durum* Desf.), which is very low, can be greatly increased by previously adding acetone-extracted lipids from hexaploid wheat (Fernández de Caleya et al., 1976; Hernández-Lucas et al., 1977a; Hernández-Lucas et al., 1977b). We report here the extraction of a rye purothionin homologue (RPH) using a similar procedure, its purification, and its amino acid composition.

MATERIALS AND METHODS

Biological Materials. Rye, *Secale cereale* L., INIA C/171-M, and hexaploid wheat, *Triticum aestivum* L., cv Aragon 03, were the gift of Dr. J. V. Torres (Instituto Nacional de Investigaciones Agrarias, Madrid, Spain). They were milled without preconditioning in a Brabender Quadrumat mill, and 60–65% yields of milled endosperm (flour) were obtained.

Extraction Procedures. Wheat flour (500 g) was extracted with four volumes of acetone in a glass column (10 × 40 cm), and the solvent was evaporated under vacuo. This extract was redissolved in approximately 500 mL of petroleum–ether (bp 50–70 °C) and mixed thoroughly with 500 g of rye flour, and the solvent was allowed to evaporate overnight. The flour was then placed again in the glass column and extracted with 4 volumes of petroleum–ether. This extract was concentrated under vacuo at room temperature to about 10 mL and treated with 1 N HCl in ethanol, and the precipitate formed, collected by centrifugation, as previously described (García-Olmedo et al., 1968). To serve as controls, the petroleum–ether extract from untreated rye flour (500 g) and the acetone extract from wheat flour (500 g) were also treated with 1 N HCl in ethanol.

Sulfuric acid extraction of RPH was as reported by Fernández de Caleya et al. (1976) for the wheat purothionins.

Purification. RPH was purified from the petroleum–ether precipitate by preparative electrophoresis on 10% polyacrylamide columns (1.5 × 10 cm) with 0.1 M acetic acid buffer pH 2.9, at 20 V/cm, as described for wheat purothionins (Hernández-Lucas et al., 1974). An electro-elution adaptor, similar to that described by Popescu et al. (1971), was used to collect the protein fractions, which were directly freeze-dried. A mixture of α- and β-purothionins from hexaploid wheat was similarly prepared.

For purification control, and as an assay method, one- and two-dimensional starch gel electrophoresis were used as previously described (Fernández de Caleya et al., 1976).

Amino Acid Analysis. Amino acid analysis of RPH was carried out essentially as described by Moore and Stein (1963). Appropriate aliquots were hydrolyzed for 24 and 48 h in a thermoblock at 110 ± 1 °C. A Jeol JLC-6AH autoanalyzer was used.

RESULTS AND DISCUSSION

Rye flour, to which acetone-extracted lipids from an equivalent amount of wheat flour had been added, yielded 97 mg of precipitate/kg, by petroleum–ether extraction followed by treatment with 1 N HCl in ethanol. Untreated rye flour yielded only trace amounts of precipitate, and none was obtained from the acetone extract of wheat. The precipitate was fractionated by starch gel electrophoresis and a prominent band with the same mobility as wheat β-purothionin was observed (Figure 1). This was confirmed by two-dimensional electrophoresis (Figure 2).

We have previously demonstrated that wheat purothionins are also extracted with 0.05 N H_2SO_4 (Fernández

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Table I. Amino Acid Composition of the Rye Purothionin Homologue (RPH) and of β-Purothionins from Different Wheat Species

Amino acid	RPH		Wheat β-purothionins			
					T. aestivum	
	Found	Nearest integer	*T. durum*[a]	*T. monococcum*[a]	*a*	*b,c*
Lysine	4.5	5	5	5	5	6
Arginine	4.3	4	3	3	3	4
Aspartic	2.8	3	3	4	4	4
Threonine	2.2	2	2	2	2	2
Serine	4.2	4	4	4	4	4
Glutamic	2.8	3	2	2	2	1
Proline	1.7	2	2	2	2	2
Glycine	4.2	4	4	3	3	3
Alanine	2.3	2	3	3	3	3
Valine	0.4		1	1	1	1
Isoleucine	1.1	1				
Leucine	2.9	3	4	4	4	5
Tyrosine	0.7	1	1	1	1	1
Phenylalanine	1.4	1	1	1	1	1
Half-cystine	*d*	*d*	8	8	8	8
No. of residues		43	43	43	43	45
Molecular weight		4720[d]	4605	4663	4663	4931

[a] Fernández de Caleya et al. (1976). [b] Ohtani et al. (1975). [c] Mak and Jones (1976b). [d] Extensively destroyed during hydrolysis; a nearest integer of 8 was assumed for the molecular weight calculation.

Figure 1. Densitogram of starch gel electrophoresis (0.015 M aluminum lactate–lactic acid buffer, 3 M urea, pH 3.2) of precipitate obtained from the petroleum–ether extract of rye flour after previous addition of the wheat acetone extract (——). Idem of α and β wheat purothionins (------).

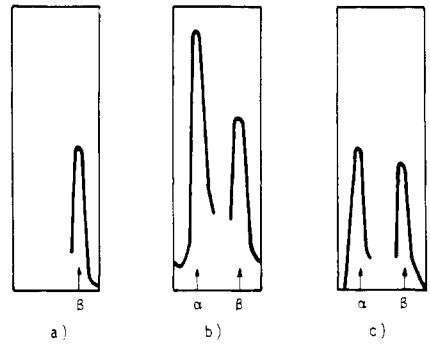

Figure 2. Densitograms of starch gel two-dimensional electrophoresis (0.02 M sodium acetate buffer, pH 5.2 × 0.015 M aluminum lactate buffer, 3 M urea, pH 3.2): (a) rye purothionin homologue (RPH) extracted with petroleum–ether after addition of the wheat acetone extract; (b) α and β wheat purothionins; (c) mixture of a and b.

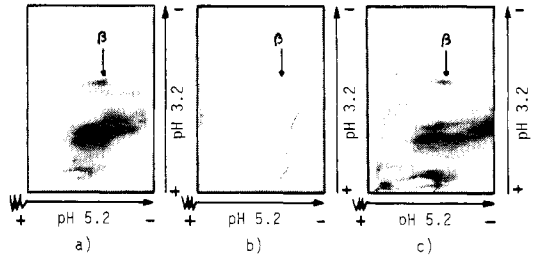

Figure 3. Two-dimensional electrophoresis of RPH: (a) protein extracted from rye with 0.05 N H_2SO_4; (b) protein extracted from rye with petroleum–ether after addition of the wheat acetone extract; (c) mixture of a and b. First electrophoresis: 0.02 M sodium acetate buffer, pH 5.2; second electrophoresis: 0.015 M aluminum lactate buffer, 3 M urea, pH 3.2.

de Caleya et al., 1976). Figure 3 shows that RPH is similarly extracted with dilute sulfuric acid.

Electrophoretically homogeneous RPH was obtained from the precipitate by preparative electrophoresis. The amino acid composition of the purified RPH is presented in Table I. Previously reported amino acid analysis of β-purothionins from different wheat species are also included for comparison (Fernández de Caleya et al., 1976; Ohtani et al., 1975; Mak and Jones, 1976b). RPH composition is closer to that reported by us for β-purothionin from *T. durum* (+1 arginine, +1 glutamic, +1 isoleucine; −1 alanine, −1 valine, −1 leucine) than to that from either *T. aestivum* or *T. monococcum*, which has an additional substitution (+1 glycine; −1 aspartic).

Ohtani et al. (1975) have found, for what they call purothionin A-1, the same sequence of 45 residues as that reported by Mak and Jones (1976b) for the β-purothionin from *T. aestivum*. In this case, the differences of RPH are somewhat greater.

These results demonstrate the long suspected presence in rye of a homologue of wheat purothionin. A genetic study on the chromosomal location of the structural gene for this protein (RPH), to be published elsewhere, fully confirms this finding.

The fact that the concentration of RPH in rye is of the same order as that of total purothionin in wheat and that rye lacks petroleum–ether soluble RPH further supports the previous suspicion that the lipid–protein complexes, extracted with petroleum–ether from the endosperm of wheat and some other Gramineae, are extraction artifacts

and may not exist as such "in vivo".

The purothionin homologue from barley was named "hordothionin" by Redman and Fisher (1969). Accordingly, the rye homologue described in this paper could be designated "secalethionin". However, this class of proteins seems to be more widely distributed in the plant kingdom than previously suspected, since they have been already found in species of the genera *Aegilops*, *Triticum*, *Hordeum*, and *Secale*, as well as in a species as phylogenetically distant from the Gramineae as the European mistletoe (*Viscum album*, L.). Moreover, several species might have the same genetic variant. For these reasons, the present nomenclature does not seem to be adequate. Therefore, we propose the general designation of *thionins* for this type of proteins, followed by the name of the species from which it has been isolated, and by a greek letter, if more than one variant is present in a given species.

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Digestibility and Lysine Values of Proteins Heated with Formaldehyde or Glucose

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Experiments were done with a blend of lactalbumin and ovalbumin with ca. 13% moisture content. Heating the protein for 2 h at 80 °C with either 1% formaldehyde or 6% glucose (to supply the equivalent quantity of carbonyl groups) resulted in approximately a 15% loss of total lysine and 25–35% decrease in reactive lysine in either case. Neither product appeared toxic, but the protein heated with HCHO showed a 25% decline in digestibility and a 50–60% decrease in "available lysine" value for both rats and chicks. The protein heated with glucose showed unchanged digestibility and only a 30% decrease in available lysine value. The more general damage with HCHO is consistent with it reacting first with lysine and then forming cross-linkages by the Mannich reaction. It confirms the need for caution in using HCHO to preserve or harden animal protein materials prior to drying.

In an earlier study comparing fish meals prepared in different ways (Carpenter and Opstvedt, 1976), the application of formalin as a firming agent immediately prior to processing, to ease the process of pressing liquor out of the fish after cooking, gave the poorest product as judged by growth assays with rats or chicks with lysine as the limiting amino acid. This inferiority, however, was not predicted by chemical tests. When glucose had been added to proteins before processing the same chemical tests had proved sensitive detectors of damage to lysine (Hurrell and Carpenter, 1974). Since both chemicals, i.e., formaldehyde and glucose, are thought to react with proteins by virtue of their carbonyl group, it might be thought that they would have similar effects. The present study was designed to investigate this by direct comparison.

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MATERIALS AND METHODS

Test Materials. Two-hundred-gram portions of a mixture of ovalbumin and lactalbumin (3:2 by weight) (cf. Hurrell and Carpenter, 1974) were added to 200 mL of formaldehyde solution containing either 0.75, 1.0, or 1.5% formaldehyde or to 200 mL of a 6% glucose solution. The pH of all mixes was approximately 5.2. Each slurry was mixed for 1 h in a food mixer, freeze-dried, and then ground in a hammer mill with a 1-mm screen. The freeze-dried materials were adjusted to 15% moisture before sealing into McCartney bottles which were stored immediately at −20 °C (control), or either heated in an oven for 2 h at 80 °C or held for 5 days at 37 °C before being stored at −20 °C. The intermediate concentration

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