External Association of Hordothionin with Protein Bodies in Mature Barley

Pilar Carbonero,* Francisco Garcia-Olmedo, and Carlos Hernandez-Lucas

Hordothionin, a high sulfur basic polypeptide from barley endosperm, cosediments with protein bodies (hordeins) during sucrose gradient centrifugation. Hordothionin can be quantitatively extracted with 0.05 N H_2SO_4 from that particulate fraction without apparently affecting the protein body structure, while hordeins can be extracted with 55% 2-propanol, 2% mercaptoethanol, without extracting hordothionin, which is soluble in this solvent. It is concluded that hordothionin is integrated in the protein–lipid matrix in which protein bodies are embedded in mature barley endosperm.

The thionins are basic polypeptides with molecular weights of ~5000 and a high cysteine content which have been found in the endosperms of wheat (Balls and Hale, 1940; Balls et al., 1942a,b), barley (Redman and Fisher, 1969), rye (Hernandez-Lucas et al., 1978), and in 22 species of the *Aegilops–Triticum* group (Carbonero and Garcia-Olmedo, 1969). Their physical and chemical properties have been extensively studied (Garcia-Olmedo et al., 1968; Nimmo et al., 1968; Fisher et al., 1968; Redman and Fisher, 1968; Nimmo et al., 1974; Hernandez-Lucas et al., 1977a,b), and several genetic variants have been described (Fernandez de Caleya et al., 1976; Sanchez-Monge et al., 1979), some of which have been sequenced (Mak and Jones, 1976a,b; Jones and Mak, 1977; Ohtani et al., 1975, 1977). They were first discovered as protein–lipid complexes in petroleum–ether extracts from wheat flour and were designated purothionins (Balls and Hale, 1940; Balls et al., 1942a). We have shown that only a fraction of the thionins is extractable with petroleum ether and that digalactosyl diglyceride binding is required for solubility (Hernández-Lucas et al., 1977b). Little is known about their physiological function and location within the starchy endosperm. In this context, we now present evidence that a substantial fraction of the thionin from mature barley (hordothionin) is externally associated with protein bodies and is an important component of the protein–lipid matrix in which the protein bodies are embedded.

MATERIALS AND METHODS

Barley Endosperm. Barley cv. Zephyr was used throughout this study. A 2-kg sample was passed twice through a rice mill, eliminating the embryo, the pericarp, and the aleurone layer (25% dry matter loss), and the resulting endosperm was milled through a Culatti mill (fine setting).

Protein Body Preparation. In preliminary experiments, protein bodies were prepared according to Ory and Henningsen (1969). In subsequent experiments a modification of the Larkins and Hurkman (1978) procedure was followed: the milled endosperm was homogenized with mortar and pestle for about 5 min at 4 °C in 3–4 volumes of Tris buffer [20 mM tris(hydroxymethyl)aminomethane, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, adjusted to pH 8.5 with HCl] containing 0.2 M sucrose. The homogenate was filtered through four layers of cheesecloth and centrifuged at 500*g* for 5 min. The supernatant was layered on top of continuous or discontinuous sucrose gradients made in Tris buffer (see legends of Figures) and ultracentrifuged at 75000*g* for 1 h in the SW 41 rotor of a Beckman L3-40 ultracentrifuge. Collected fractions were diluted with buffer, quantitated spectrophotometrically at 280 nm, and pelleted at 20000*g* for 30 min in conical 10-mL tubes.

Selective Solubilization of Hordothionin. The fraction collected at the 50–70% (w/v) interface of a discontinuous sucrose gradient, designated protein body fraction (PB fraction), was used in the survey of conditions for the selective solubilization of hordothionin. Aliquots of the PB fraction were diluted with 3 volumes of a solution of the agent to be tested: 0.05 N H_2SO_4, 1 M NaCl, 1% (v/v) Nonidet P-40 (NP-40), 0.2 N NaOH, 0.5 N acetic acid, 1% sodium metabisulfite, 1% sodium cholate, or 1% sodium deoxycholate. After incubation at 4 °C for 30 min, the suspensions were either directly centrifuged at 36000*g* for 10 min or layered on top of discontinuous gradients (see legends of Figures).

Analytical Procedures. The pelleted gradient fractions were freeze-dried prior to analysis.

Lipids were extracted with acetone (500 μL + 500 μL) in the conical centrifuge tubes. Acetone was used in order not to extract thionin (Hernandez-Lucas et al., 1977b). The pellets were sonicated for 30 s in a Branson Sonifier B-12 (output set at 1), allowed to stand at room temperature for 1 h, and centrifuged at 3000*g* 5 min. The solvent from the combined extracts was evaporated in vacuo in 3-mL tubes and the extracts were redissolved in 50 μL of chloroform. Appropriate aliquots were applied to thin-layer plates precoated with silica gel 60 (Merck). Chromatography of nonpolar lipids was carried out with petroleum ether/diethyl ether/acetic acid (65:36:1, v/v/v) and that of polar lipids with chloroform/methanol/water (65:25:4, v/v/v). After chromatography, the plates were either exposed to iodine vapor or sprayed with 50% H_2SO_4 and briefly heated at 100 °C to estimate digalactosyl diglyceride. Known amounts of acetone extracted lipids from barley endosperm were used as standards for the densitometric determinations.

Hordothionin quantitation was carried out in the delipidated pellets. These were dispersed with sonication in 100 μL of 0.015 M aluminum lactate, pH 3.2, 8 M urea buffer, and 20-μL aliquots were applied to starch gels made with the same buffer in 3 M urea. Electrophoresis was performed at 10 V/cm for 1 h. Densitometric quantitation was carried out using electrophoretically homogeneous hordothionin as standard, which was obtained by preparative electrophoresis as previously described for purothionins (Hernandez-Lucas et al., 1974).

Hordeins were extracted from 50 μL of the above dispersion with 500 μL of 55% 2-propanol, 2% mercaptoethanol, at 60 °C for 1 h with sonication, essentially as described by Shewry et al. (1977). The solvent was evaporated in vacuo and the extracts electrophoresed according to Laemli (1970), using a 10% to 25% acrylamide gradient slab. This procedure gave the same yield and electrophoretic pattern as direct hordein extraction from

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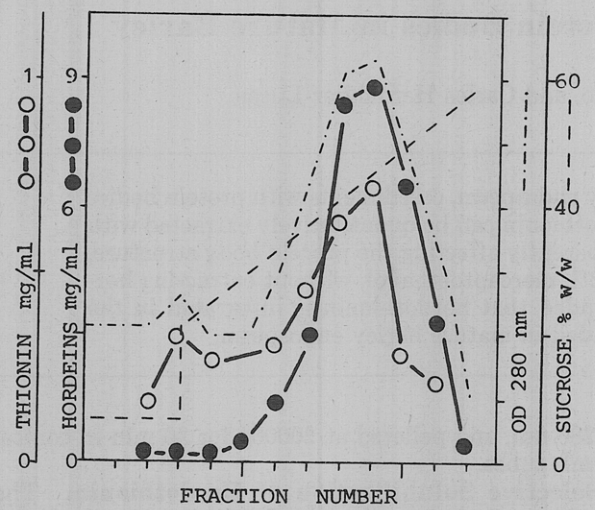

Figure 1. Distribution of thionin (O–O) and hordeins (●–●) after centrifugation in a 20%–60% (w/w) continuous sucrose gradient of a 500*g* supernatant of a barley endosperm homogenate. Absorbance at 280 nm (---) and sucrose concentration (w/w) (- -) are also recorded.

the pellets. Densitometric determinations were carried out using as standards Zephyr hordeins obtained by the sequential extraction procedure of Shewry et al. (1977).

In all cases, densitometry was performed with a Chromoscan densitometer (Joyce & Loebl) with a 620-nm filter. Values of unknowns were obtained by interpolation between the nearest standard concentrations (SE ≤ 10%).

Microscopical Observations. To detect the possible contamination of protein body preparations by starch granules, the samples were treated with I_2–KI and examined under the light microscope.

Scanning electron microscopy (SEM) was carried out in an ISI minimicroscope. A suspension of the sample was frozen on top of a specimen holder with a double-coated adhesive tape, freeze-dried, and coated with platinum–palladium, prior to observation.

RESULTS

In preliminary experiments, high levels of thionin were found in preparations of protein bodies (PB) from mature barley endosperms obtained as described by Ory and Henningsen (1969). To investigate further the possible association of thionin with protein bodies, the supernatant from a centrifugation at 500*g* of an endosperm homogenate was analyzed by continuous sucrose density gradient centrifugation. As shown in Figure 1, the hordein and the thionin peaks did coincide, although about one-third of the thionin was present in lower density fractions that have little or no hordein content.

The PB fraction had the following approximate composition: hordein (50–60%), thionin (3–4%), other proteins (15–20%), and acetone-extracted lipids (18–22%). No significant differences were found between the thin-layer patterns of polar and nonpolar lipids from PB and those from whole flour lipids, which were used as standards (Figure 2). The thionin/hordein ratio in PB is about twice that in whole flour.

Selective solubilization of thionin from the PB fraction was attempted under mild conditions (30 min at 4 °C) using a variety of agents: 0.05 N H_2SO_4, 1 M NaCl, 1% NP-40, 0.2 N NaOH, 0.5 N acetic acid, 1% sodium metabisulfite, 1% sodium cholate, and 1% sodium deoxycholate. After incubation, the particulate fraction was pelleted, and both the pellet and the supernatant were analyzed for hordeins and thionin. Only negligible amounts of hordeins were present in the supernatants

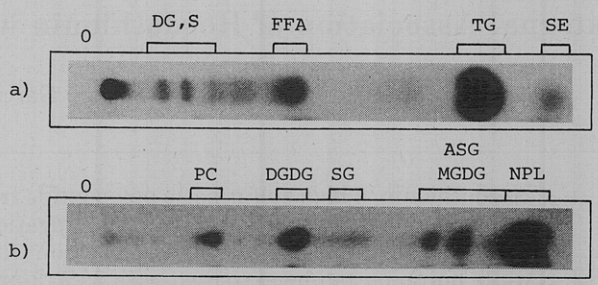

Figure 2. Thin-layer chromatography of acetone-extracted barley lipids. (a) Separation of nonpolar fractions with petroleum ether, diethyl ether, acetic acid (65:36:1, v/v/v): sterol esters (SE), triglycerides (TG), free fatty acids (FFA), diglycerides (DG), sterols (S). (b) Separation of polar fractions with chloroform–methanol–water (65:25:4, v/v/v): nonpolar lipids (NPL), acylated steryl glycosides (ASG), monogalactosyl diglyceride (MGDG), steryl glycosides (SG), digalactosyl diglycerides (DGDG), and phosphatidyl choline (PC).

obtained from the first three solvents. Thionin was quantitatively extracted with 0.05 N H_2SO_4, partially extracted with 1 M NaCl, and not extracted with 1% NP-40 (Figure 3b). However, purified thionin was readily soluble in 1% NP-40. The remaining treatments resulted in the solubilization of a substantial fraction (>30%) of the hordeins, indicating disruption of protein bodies.

When hordeins were extracted from the PB fraction with 55% 2-propanol, 2% mercaptoethanol, at 60 °C, with sonication (Figure 3a), thionin remained in the pellet and was converted to the reduced form (Figure 3b) although purified thionin is readily soluble in this solvent.

The effects of treatments with sulfuric acid and with NP-40 were further investigated by density gradient centrifugation (Figure 4). The sedimentation profile of hordeins was slightly displaced toward lower density after sulfuric acid treatment and significantly toward higher density when treated with NP-40. Thionin cosedimented with hordeins in the NP-40 treated sample. The greater density of this sample is probably due to the observed extraction of lipids by the detergent, and the lower density of the sulfuric acid treated protein bodies can be explained by the selective extraction of thionin by this agent.

Negligible amounts of starch granules were present in the PB fraction, as judged by light microscopy of I_2–KI stained preparations. Protein bodies appeared to be embedded in a matrix when observed by SEM, and their spherical form was barely discernible (Figure 5a), whereas most of the matrix was absent, and the protein bodies were clearly outlined after the sulfuric acid treatment (Figure 5b).

DISCUSSION

The present results indicate that hordothionin is quite abundant in mature barley endosperm. If the difference in molecular weight with respect to the major hordeins (5000 vs. 30 000–90 000) and the fact that there are at least ten hordein components are taken into account, it can be concluded that, on a molar basis, hordothionin is at least as abundant as individual components of the reserve protein.

The observed higher thionin/hordein ratio in PB as compared with whole endosperm could be due either to a lower extraction efficiency of thionin from endosperm or to an actual enrichment of thionin vs. hordein in the PB preparation.

It should be mentioned that hordothionin, which has 12 mol of lysine/100 amino acid residues (average for endosperm protein is 4–5 mol %), is increased about 50% in the high lysine mutant Riso 1508 and decreased about 40%

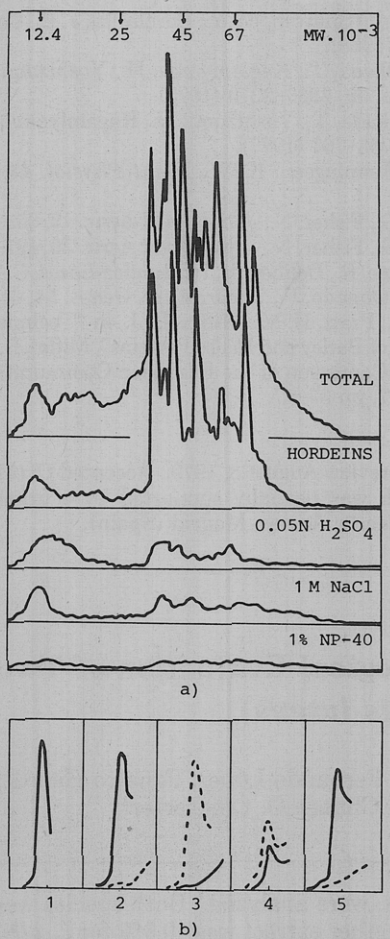

Figure 3. Selective solubilization of hordothionin from a preparation of protein bodies (PB fraction). (a) "Total" proteins from the PB fraction were extracted with the sample buffer of Laemli (1970) and hordeins with 55% 2-propanol, 2% mercaptoethanol, at 60 °C (Shewry et al., 1977). Proteins present in the supernatant after treatment with either 0.05 N H_2SO_4, 1 M NaCl, or 1% (w/v) NP-40 and centrifugation (36000*g*, 10 min) were precipitated with 12% Cl_3CCOOH. All samples were electrophoresed in the same slab by the method of Laemli (1970) with a 10%–25% acrylamide gradient and compared densitometrically. (b) Hordothionin in pellets (–) and supernatants (- -) after centrifugation (36000*g*, 10 min) of the PB-treated preparation was determined by starch gel electrophoresis in aluminum lactate buffer, pH 3.2: total in PB fraction (1), after 55% 2-propanol, 2% mercaptoethanol treatment (2), same as above after 0.05 N H_2SO_4 (3), same as above after 1 M NaCl (4), same as above after 1% (w/v) NP-40 (5).

in the low lysine mutant Riso 10 (Carbonero, Garcia-Olmedo, Hernandez-Lucas, unpublished results).

Several workers (Munck and von Wettstein, 1975; Ingversen, 1975; Mifflin and Shewry, 1978) have reported that protein body preparations contain homogeneously structured spheres embedded in a granular matrix. Aqueous 2-propanol, which extracts hordeins, does not extract thionin although the purified protein is readily soluble in the solvent, and, on the other hand, dilute sulfuric acid quantitatively extracts thionin without apparently affecting the protein bodies. So it must be concluded that hordothionin is externally associated with the protein bodies and is part of the protein–lipid matrix in which they are embedded. Both Larkins and Burkman (1978) in developing maize and Shewry and Miflin (1978) in developing barley have indicated that part of the endoplasmic reticulum remains associated with the protein bodies upon gradient centrifugation. These membranes would become more tightly packed around protein bodies as the kernel dries and their components would be more

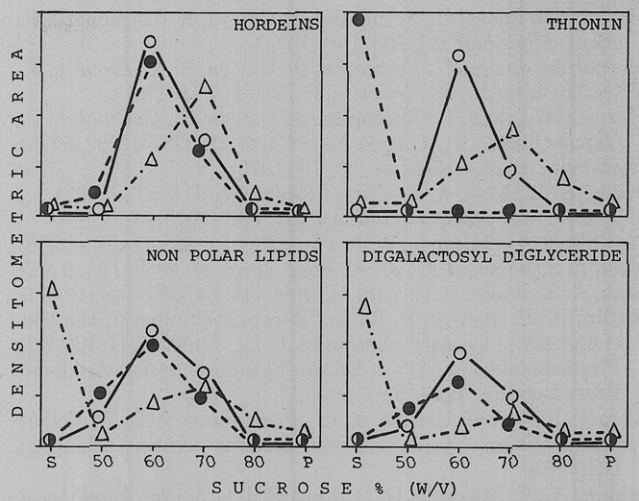

Figure 4. Effects of 0.05 N H_2SO_4 and of 1% NP-40 treatments on PB preparation. Distribution of hordeins, thionin, nonpolar lipids, and digalactosyl diglyceride in a discontinuous 50%–60%–70%–80% (w–v) sucrose gradient. Control (○–○), 0.05 N H_2SO_4 treated PB (●–●), 1% NP-40 treated PB (△–△). P, pellet. S, supernatant.

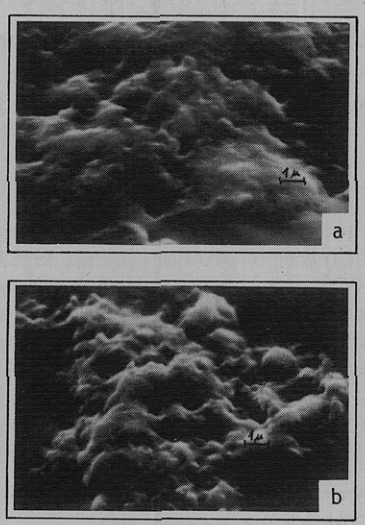

Figure 5. Scanning electron micrographs of PB preparations from barley endosperm: (a) control, (b) after sulfuric acid treatment.

difficult to extract with NP-40 in mature than in developing barley. So the possibility that hordothionin is part of the endoplasmic reticulum remains open until similar studies with developing barley endosperm are completed.

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Chemical Composition, Nutritive Value, and Toxicological Evaluation of Two Species of Sweet Lupine (*Lupinus albus* and *Lupinus luteus*)

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Two species of sweet Lupine, *Lupinus albus* and *Lupinus luteus*, were analyzed. Both species were good sources of protein (34 and 39%). Lipid content measured as ether extract was 10.9% for *L. albus* and 4.7% for *L. luteus*. Both legumes had high crude fiber contents of more than 10% and low alkaloid contents (0.05 and 0.09%). The protein efficiency ratio was low in both species (0.48 and 0.99), but supplementation with DL-methionine increased base values significantly ($p < 0.01$), 2.84 and 2.30, respectively. In a toxicity study with rats that consumed a 20% lupine protein diet (supplemented with 0.3% DL-methionine), the growth rate of animals fed *L. luteus* and *L. albus* was similar to that produced by an unsupplemented 20% protein casein diet. The weight of liver, kidneys, heart, spleen, and adrenals and the histology of kidneys and lungs were normal.

For centuries legumes have been an important source of protein and calories for many peoples of the world. Lupines among the legumes were used as a human food by ancient cultures surrounding the Mediterranean and by those people living in the Andes highlands (Grindley and Akour, 1955; Castillo, 1965), and among the vegetable crops legume grains contain the highest percentage of protein. However, the proteins of legumes are generally considered good sources of lysine, and generally low in the sulfur-containing amino acids. Some lupines referred to here as "bitter lupines" also contain high levels of the alkaloids lupanine and spartein which impart a bitter taste. Sweet lupines, or the low alkaloid species, have been developed by genetic selection (Gladstones, 1972; von Baer, 1972) and because their composition compares favorably with soybeans, sweet lupines could become an important source of protein and oil for human diets since their taste is acceptable. Their high seed yield and good growing and harvesting characteristics also make them suitable for cultivation in many areas of the world.

Different native and genetically selected lupine cultivars have been used as sources of protein in animal feeding trials with rabbits, pigs, and broilers (Flores, 1970; Pearson and Carr, 1977; Yule and McBride, 1976). The animals' productive responses were, for the most part, adequate.

In Chile, efforts are currently underway to introduce lupine flour into substitute milk formulas for infants and into various common foods. This prompted us to evaluate the chemical, nutritional, and toxicological properties of two species of sweet lupines grown in Chile: *Lupinus albus* and *Lupinus luteus*. Presented here are the results of this investigation.

MATERIALS AND METHODS

Samples of *L. luteus* and *L. albus* obtained from a local grower were ground with a laboratory hammer mill (Wiley Laboratory Standard Model 4) and passed through a 100-mesh sieve. Moisture, ether extract, total ash, and crude fiber were determined according to AOAC (1970) methods. Nitrogen was determined by a macroKjeldahl procedure, followed by a semimicrodistillation into a 2% boric acid solution with a mixed indicator (Markham,

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