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CHARACTERIZATION OF THE FINE STRUCTURE AND PROTEINS FROM BARLEY PROTEIN BODIES*

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Abstract—The fine structure and the membranes surrounding barley protein bodies have been separated from the proteins stored within the particles by sonication. The two fractions obtained have been characterized by electron microscopy and by immunoelectrophoretic analysis. Phytase activity was shown to be associated with the fine structure, and hordein was identified as the aleurin inside the protein bodies. The localization of phytic acid is discussed

INTRODUCTION

THE PRESENCE of reserve proteins in protein bodies, or aleurone grains, of several seeds is well established, and these organelles have been morphologically described by electron microscopic techniques.[1–6] The particles from oilseeds seem to contain additional substructures within the membrane, such as crystalloids or globoids.[5,7] The globoids were shown to be sites of phytic acid storage in peanuts[8] and in cotton seed.[9]

In a previous paper,[6] protein bodies isolated from dormant barley seeds were shown to have a fine structure of orderly, or lamellar, electron-transparent and electron-dense layers of some material(s) other than just storage proteins attached to the particles. Mitsuda *et al.*[10] have reported analogous structures completely within rice protein bodies. In contrast, the fine structure of the barley particles, in most cases, seemed to be an appendage associated with the membrane rather than to be within the organelle. The electron-dense layers revealed by osmic acid staining suggested that the fine structure of barley protein bodies might be the site of phytic acid storage. Finding a substrate-specific phytase associated with the barley protein bodies[6] strengthened this theory and suggested the possibility that these fine structures might also contain layers of enzymes. Vigil[11] has reported enzyme activity

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in analogous layered-type structures within microbodies isolated from endosperm tissue of germinated castor beans.

With the isolation of intact protein bodies it was now possible to answer an additional question. There are two groups of reserve proteins in barley: glutelin and hordein. Since the nature of the protein within the barley protein bodies had not been previously characterized, separation of the membranes and fine structure would also permit characterization of the reserve protein within the particles. Of the two reserve proteins in peanut, arachin and conarachin, only arachin exists within the protein body;[12] conarachin is a cytoplasmic protein. According to the concept of Altschul[13] that proteins within the protein bodies, or aleurone grains, should be classified as 'aleurins', only arachin is an aleurin.

In the previous report,[6] the fine structure appeared to be separated from the surface of the protein body in some cases, suggesting that it might be possible to remove this fraction for further characterization of its proteins and those proteins within the particle. In the present investigation the fine structure and the particle membranes have been separated from the storage protein(s) by sonication. Proteins in the two fractions were characterized using the highly specific immunoelectrophoretic analysis technique of Grabar and Williams[14] Hordein was identified as the reserve protein in the particle and phytase activity has been localized in the fine structure

RESULTS

Disruption of the protein body membrane was the first step in isolation of the fine structure. The 10-sec sonication procedure as used to rupture the membranes of mitochondria[15] was tried for the protein bodies but without effect, suggesting that these plant particle membranes might be more stable. To determine the minimum time necessary to disrupt the membranes, the protein bodies were sonicated for 20, 40, 60 and 90 sec periods in 75% ethanol and the precipitates and alcoholic solutions analyzed by immunodiffusion. The first precipitin arcs appeared after sonication for 40 sec at 20 Kc/sec, indicating this as the minimum time required to break the protein body membranes and release the contents into the alcohol.

In the preliminary sonication tests in sodium chloride solutions, immunoelectrophoretic analysis of the remaining precipitate vs. antihordein serum showed the precipitate to contain hordein. It was therefore felt that, if the reserve protein material of the protein bodies did consist of hordein, this should go into solution when sonicated in 75% ethanol and the remaining membranes and the fine structure should precipitate. The two fractions, precipitate I and precipitate II, obtained after the first sonication were subsequently fixed in glutaraldehyde and osmium acid, then examined by electron microscopy to determine the stage of disruption of the particles and the natures of the two fractions.

When protein bodies were sonicated only once, a precipitate of apparently unbroken particles but largely without the fine structure (precipitate I) remained (Fig. 1d). The second fraction obtained by centrifugation of the alcoholic supernatant (precipitate II) appeared to consist mainly of pieces of electron-dense fragments (Fig. 1c) very much like the fine structure associated with the protein bodies (Fig. 1a and b), and with relatively fewer intact protein bodies.

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To further characterize the proteins of the fine structure and the proteins stored within the particles, the three fractions, precipitate I, precipitate II, and the remaining alcoholic solution, were analyzed by immunoelectrophoretic analysis (Figs. 2 and 3) and by double diffusion (Fig. 4).

The phosphate compositions of the intact protein bodies, the fine structure fraction and the alcohol-soluble fraction are shown in Table 1.

TABLE 1. PHOSPHATE CONTENT OF BARLEY PROTEIN BODIES, THE FINE STRUCTURE FRACTION, AND THE HORDEIN FRACTION: PERCENTAGES ARE BASED ON THE DRY WEIGHT OF MATERIAL

Material	Total phosphorus μM/mg	
	Preparation 1	Preparation 2
Intact protein bodies	0·54	0·46
Hordein fraction	0 93	0·94*
Fine structure fraction	0 23	0·28

* Phosphorus determination of the hordein fraction in preparation 2 was made after inorganic phosphate was removed with $CaCl_2$.

The experiments to localize the site of phytase activity in protein bodies were conducted on pieces of aleurone tissue instead of on isolated particles, because of the low percentage of protein bodies with fine structure in an isolated preparation and because separated pieces of fine structure might make the results difficult to interpret. The results are shown in Fig. 5.

A section of aleurone layer showing protein bodies stained with potassium permanganate (Fig. 5a) is compared to a similar piece of tissue which has no stain other than the electron-dense deposits of lead phosphate located at the sites of enzyme activity (Fig. 5b). The close similarity of the electron-dense deposits of the two electron micrographs suggests that phytase activity appears to be associated with the fine structure around the protein bodies.

DISCUSSION

From Fig. 1 it is evident that the fine structure is separated from the rest of the particle by sonication in alcohol. Figure 1d illustrates that one sonication is insufficient to completely disrupt all of the larger particles. It should be noted, however, that some of the fine structure can be stripped from the particles by this first treatment. From these results it appears that sonication takes off the fine structure in stages. The first 40 sec treatment probably removes most of it and breaks some of the smaller particle membranes (Fig. 1c), leaving the larger protein bodies intact with membrane only (Fig. 1d). The second and third sonications disrupt the remaining membranes releasing storage protein(s) into the alcohol.

Hordein, the major reserve protein of barley, has been defined by its solubility in 75% alcohol based on the historical terminology of Osborne.[16] The data obtained by immunochemical analysis (Figs. 3 and 4) indicate that hordein(s) may now be classified as an 'aleurin'. The site of storage of glutelin(s), the other reserve protein of barley, cannot be

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determined from the results described here and is still unknown. It may possibly be cytoplasmic as is the conarachin in peanut.[12]

At least seven proteins (albumins and globulins) were detected by immunoelectrophoretic analysis of the isolated fine structure, according to their antigenicities and their electrophoretic mobilities. This plus the earlier finding of two acid hydrolases, phytase and protease, associated with the protein bodies in barley[6] suggests that this fine structure may contain some or all of the enzyme activities associated with these particles. The electron microscopic localization of phytase activity in the fine structure (Fig. 5) would tend to support this. It should be noted in Fig. 2 that the patterns of proteins from the isolated protein bodies and from the fine structure obtained from them are very much alike. This is a clear indication that there was no serious destruction of the proteins by three 40-sec sonications in alcohol, and confirms the findings of Prudhomme[17] regarding sonication in aqueous and non-aqueous media.

Protein bodies from various seeds are known to contain a high amount of phytate[1,8,9] The earlier results on isolated protein bodies of barley[6] indicated that phytate might be located in this fine structure It was proposed that the darker alternate layers could be phytic acid rendered electron-dense by the osmium binding to phytate anions. However, if this were so, sonication should concentrate the phytic acid in the fine structure fraction. Phosphorus analysis of the two fractions after sonication shows the phosphorus content to be highest in the hordein fraction. This, plus the observation that the hordein fraction constitutes a major part of the protein bodies (about 70%), suggests that this fraction contains the major proportion of the phosphorus. This apparent high phosphorus content in the hordein fraction does not rule out the proposal that phytate is stored in the fine structure, but it opens the possibility that phytate in barley protein bodies is also distributed with the hordein and does not exist in specific areas as globoids, the concentrated forms of phytate in peanut[8] and cottonseed protein bodies[9] Saio *et al.*[18] have shown protein–phytic acid binding in soybean, and Tombs[1] has demonstrated that the phytic acid in soybean is presumably bound to glycinin. It is also possible that sonication splits parts of the fine structure releasing some phytate into solution in 75% ethanol, and the *in vitro* evidence obtained here on the very small quantities of material available may not describe the *in vivo* conditions exactly Therefore, a specific site of phytate storage in barley protein bodies cannot be conclusively stated from these data.

EXPERIMENTAL

Materials and Methods

Seed source. Barley seeds, two row, *Hordeum vulgare*, var. Kenia, were a gift from the Carlsberg Brewery Research Laboratory, Copenhagen, Denmark

Isolation of protein bodies. Protein bodies were isolated by a combination of buffer-extraction and centrifugation over sucrose gradients as previously described.[6] For phosphate analysis the protein bodies were rinsed in isotonic sodium chloride, and the precipitate of centrifuged protein bodies dried for three days over P_2O_5 in a dessicator.

Sonication procedure 0 01 g of isolated protein bodies were sonicated for 40-sec intervals at 20 Kc/sec in 2 0 ml 75% ethanol in a MSE disintegrator at 6°. The samples were sonicated three times. After the first 40-sec treatment a precipitate of associated particles was formed immediately after sonication was stopped The supernatant fluid was decanted from this precipitate (precipitate I) and the unclear supernatant solution centrifuged for 15 min at 2600 *g* to yield a second precipitate (precipitate II) and a clear alcoholic supernatant solution. The two precipitates were saved and the sonication procedure was repeated on precipitate I twice more. After the third sonication nearly all of the protein bodies were disintegrated; the three alcoholic

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supernatant solutions and the three precipitates (precipitate II) were combined separately, and the supernatants were freeze dried. For phosphate analysis, precipitate II was dried for 3 days over P_2O_5 in a dessicator Before selecting 75% ethanol as the medium for sonication, 0·2 M NaCl was used. Preliminary tests by immunoelectrophoretic analysis of the precipitate and the supernatant solution identified hordein in both; a small part of the hordein being soluble in salt solutions [19] Since sonication in aqueous or dilute salt solutions was reported to destroy certain enzymes and proteins,[17] alcohol was subsequently used exclusively for the sonication experiments.

Preparation of samples for electron microscopy. The isolated protein bodies and the protein body fractions were fixed in glutaraldehyde and stained with osmic acid as described earlier [6] For potassium permanganate staining, 1 mm piece of aleurone layer from dormant barley seeds were fixed in glutaraldehyde, rinsed in buffer, and then stained in 2% aq. $KMnO_4$ for 30 min. The pieces were then washed well in water, dehydrated, and embedded for electron microscopic analysis.[6]

Localization of phytase activity Dormant barley seeds were soaked in ice water for 1 hr to soften the tissue. 1-mm pieces were dissected from the aleurone layer under a binocular microscope, incubated in 0 5% sodium phytate in pH 5·0 acetate buffer 20 min, transferred to 0 5% lead acetate at pH 5 0 for 20 min, washed well in water to remove soluble lead ions, then fixed in 4 2% glutaraldehyde in pH 7·2 veronal buffer for 2 hr at 4°. The pieces of tissue were then rinsed free of glutaraldehyde with the same buffer but with no further metal staining other than the uranyl acetate–lead citrate poststaining of the thin sections for electron microscopic analysis.[6]

Preparation of immunsera Serum against total barley proteins was prepared by subcutaneous injections of the proteins into rabbits seven times at weekly intervals [20] Two weeks after the last injection the animals were bled The antigens for injection consisted of a crude freeze-dried albumin–globulin preparation in Freund's complete adjuvant

The specific antihordein serum was a gift from the Carlsberg Brewery Research Laboratory, Copenhagen. The hordein used for these injections was extracted from barley with 75% ethanol. Possible albumins and globulins were removed by extracting this isolated crude hordein preparation by salt solution The serum was prepared in the same way as for antitotal barley, using acetic acid to dissolve the hordein. The antihordein serum showed no precipitin lines when tested against the albumin–globulin fraction from barley. Antisera were stored at −20° until used

Immunochemical analyses. Immunoelectrophoretic analysis of the fractions was carried out according to Grabar and Williams[14] as modified by Scheidegger[21] using the LKB-apparatus. The proteins were first separated by electrophoresis in a 1 25% agarose gel buffered with 0 05 M veronal, pH 8·2, and electrophoresis was carried out for 3 hr at 4° in a gradient of about 6 Vcm^{-1}. For the analyses by double diffusion the same gels and buffers were used. The immunodiffusions were complete after 3 days at 4°. For the immunoassays, 5 mg of intact protein bodies were ground in a mortar and in the cold with 0 250 ml buffer (0 1 M phosphate, 0 4 M NaCl, pH 6 6) for 5 min. The sonicated fractions were suspended in 100 μl of the same buffer.

Phosphorus determination Total phosphorus contents of the samples were determined by Bartlett's procedure [22]

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