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# Enzymes Associated With Protein Bodies Isolated From Ungerminated Barley Seeds

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*Abstract.* Protein bodies were isolated intact from dormant barley seeds, *Hordeum vulgare*, var. Kenia, by a combination of buffer extractions and centrifugations over a sucrose gradient. Examination of the protein bodies pellet in the electron microscope shows 2 types of protein bodies in a wide variation of sizes. The majority of them stain evenly with osmium, are contained within a single membrane, and have no other structural components. The other type, mostly the larger particles, has a fine structure of orderly dark and light-stained layers attached to the protein bodies. Two acid hydrolases are associated with these particles: acid phosphatase activity, specific for sodium phytate but inactive on β-glycerol phosphate, glucose 1-phosphate, fructose 1,6-diphosphate and adenosine triphosphate; and acid protease activity.

There have been several reports on the isolation of protein bodies from soybean (12, 20), various oilseeds (2, 16, 21), and cereal grains (5, 8, 9). The oilseeds seem to have additional substructures within their protein bodies. Castor beans (11) and hempseed (16) have crystalloid-type substructures which were recently isolated intact from the hempseed and shown to be pure edestin, the major storage protein. Oilseed protein bodies also contain substructures called globoids. In the peanut (2) and the cottonseed (7) these were shown to be the sites of phytic acid storage. Various acid hydrolases were found to be associated with the protein bodies of cottonseed (21) and hempseed (17).

Substructures such as crystalloids and globoids were not found in either the wheat or the rice protein bodies. Electron microscopic analysis of isolated wheat protein bodies (5) showed evenly stained particles with no further substructures, but those in developing wheat endosperm (6) contain a faint lamellar structure just within and parallel to the protein body membrane. A similar study of protein bodies in intact rice endosperm (9) showed evenly stained particles, but recently Mitsuda *et al.* (8) reported the isolation of protein bodies having a substructure of concentric rings completely within the particles. This unique structure suggests that major differences might exist between the protein-storing organelles in oilseeds and in cereal grains. No enzyme activity was reported in the cereal protein bodies.

The purpose of this investigation was to isolate protein bodies intact from ungerminated barley seeds, to determine if these particles had substructures, such as globoids or lamellar-type rings, and to see if any acid hydrolase activity was associated with them. The results described show that some barley protein bodies have a fine structure which seems to be associated with the membrane rather than entirely within it as in wheat and rice. Also, acid phosphatase specific for sodium phytate and acid protease activity were shown to be associated with the isolated particles.

## Materials and Methods

*Seed Source.* Barley seeds, 2-row, *Hordeum vulgare*, var. Kenia, were a gift from the Carlsberg Brewery Research Laboratory, Copenhagen.

*Isolation of Protein Bodies.* The following modification of the Mitsuda *et al.* (9) scheme for isolating rice protein bodies was found to yield the cleanest preparation from barley, though not necessarily a quantitative isolation. Dry barley seeds in 10 to 15 g portions were first milled for 15 sec in an EBC Mill, Casella Company, London, to pass through a 200-mesh screen. Only this bran was saved since it was found to contain less starch than a longer or complete milling of the seeds. This served as the starting material. All of the following steps were conducted at 0 to 4°.

(a) Milled barley (7 g) was ground by mortar and pestle in 21 ml of 0.1 M phosphate buffer, pH 7.2, containing 6 mM $MgCl_2$, for 4 to 5 min, then centrifuged at 350*g* for 15 min. (b) The supernatant solution was saved and the residue again extracted in 15 ml of buffer and centrifuged as in (a). The

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precipitated cell debris and starch were discarded and the 2 supernatants combined. (c) This supernatant solution was layered over 10 ml of 40 % (w/v) sucrose and centrifuged at 20,000*g* for 30 min to precipitate the protein bodies. (d) The pellet was resuspended in 15 ml of the buffer and step (c) repeated to remove any residual starch grains, mitochondria, and proplastids. For those preparations to be used in protease activity studies, in step (d) the usual phosphate-magnesium buffer also contained 0.05 M $\beta$-mercaptoethanol to protect sulfhydryl groups. (e) The final precipitate was then resuspended in 10 ml of buffer to rinse out the sucrose and centrifuged at 20,000*g* for 30 min. For electron microscopy and enzyme tests the phosphate/$MgCl_2$ buffer was used for the final rinse (containing $\beta$-mercaptoethanol for protease tests). For chemical analyses the protein bodies were rinsed in 3 mM veronal buffer and the precipitate dried for 3 days over $P_2O_5$ in a desiccator. Yield was about 1 mg per g of starting material.

*Chemical Analyses.* Total nitrogen was determined by microKjeldahl (18); total phosphorus by Bartlett's procedure (1); and total lipids by extracting the samples 3 times with 5 ml of 3:1 chloroform: methanol at 55°, twice in 5 ml ether at room temperature, and filtering each time through a sintered glass filter. Extracts were combined and the solvent evaporated under a stream of nitrogen and mild infrared heat. Both the lipids and the residues were dried over $P_2O_5$ and weighed. Total RNA was determined by the method of Schmidt and Thannhauser (14).

*Electron Microscopy.* Immediately after the final rinse, the protein bodies pellet was transferred to 4.2 % (v/v) glutaraldehyde in pH 7.2 phosphate buffer, 0.15 M, and fixed for 2 hr at 4°. It was then rinsed in 3 changes of 0.03 M veronal buffer, pH 7.4, at 4° for 1.5 hr between each rinse, and allowed to stand overnight in a fourth change of buffer. The fixed tissue was stained in 2 % (w/v) osmic acid in the veronal buffer for 2 hr at room temperature, rinsed in 4 changes of buffer, gradually dehydrated in alcohol and embedded in an epoxy resin-acid anhydride (15). Thin slices were made on a Cambridge ultramicrotome, post-stained in uranyl acetate and lead citrate, and observed in a Siemens Elmiskop I or a Zeiss EM 9A electron microscope.

*Assay of Acid Phosphatase Activity.* Protein bodies pellets (which had been stored overnight at 0° in stoppered centrifuge tubes without $P_2O_5$ drying) were macerated in 0.1 M acetate buffer, pH 5.0, in a small mortar and pestle for 2 to 3 min at 0°, and diluted to yield a final concentration of approximately 4 mg/ml, based on dry weight. This served as the enzyme source. All substrates were 5 % (w/v) solutions in 0.1 M, pH 5.0 acetate buffer. All tests contained 2 ml of the 5 % substrate solution (10 mg) and 0.5 ml enzyme (2 mg). Reactions were conducted at room temperature (22–23°) for 1 hr and terminated by adding 2 ml of 10 % (w/v) trichloroacetic acid. Blanks were obtained by adding the trichloroacetic acid just before the enzyme. The trichloroacetic acid solutions were centrifuged at 2000*g* for 15 min and 1 ml aliquots removed for analysis of enzyme-produced $P_i$ (1).

A pH 7.2 phosphate buffer extract of the total barley proteins which had been dialyzed and freeze-dried served as a crude enzyme control for comparison purposes.

*Assay of Protease Activity.* (a) BAPA-ase activity. For measuring the hydrolysis of BAPA[2] by BAPA-ase, the method of Suolinna *et al.* (19) was followed. This measures hydrolysis of BAPA by the enzyme at pH 8.6 by continuous reading of the increase in extinction at 410 m$\mu$ in a Beckman DU spectrophotometer.

(b) Acid protease activity. The method used was that described by Enari *et al.* (3). This measures the decrease in viscosity of a 5 % (w/v) gelatin (Difco, Bacto-Gelatin) in acetate buffer due to acid protease activity. In the present studies, tests were made at 33° and at pH 4.0 and 5.0.

The enzyme source was prepared by grinding the protein bodies in acetate buffer in a mortar and pestle as employed in the phosphatase tests. For those preparations used to assay for BAPA-ase activity, tris buffer, pH 7.2, replaced the acetate buffer. The total barley extract again served as a BAPA-ase control for comparison.

## Results

An electron micrograph of ungerminated barley aleurone layer is shown in Fig. 1. Organelles containing a deranged membrane system and large electron-dense bodies are frequent in the aleurone tissue. These organelles may either be a special type of protein body, or they are more than likely remnants of chloroplasts degraded during maturation of the seed.

Fig. 2 and 3 show representative areas from isolated protein body preparations. In Fig. 2 the wide variation in size of the particles is evident and they are devoid of any substructures. Most of the areas scanned in the microscope were of this type, but numerous protein bodies had appendages such as those in Fig. 3. When such structures were magnified further (insert, Fig. 2) they were found to consist of an orderly array of electron-dense and electron-transparent layers.

The composition of the protein bodies is shown in table I and acid phosphatase activity associated

[2] Abbreviation: BAPA: $\alpha$-*N*-benzoyl-DL-arginine *p*-nitroanilide.

with the particles is listed in table II. The 5 phosphate esters selected represent different substrates possibly accessible *in vivo*: a 3-carbon and a 6-carbon monoester ($\beta$-glycerol-P and glucose 1-P) a 6-carbon diester (fructose 1,6-P), a polyphosphate (ATP), and sodium phytate, which has been reported as a protein-bound energy source in a wheat amino acid incorporating system that contained protein bodies (10).

Fig. 4 illustrates the acid protease activity associated with the protein bodies measured at pH 4.0 and 5.0. Fig. 5 shows the activity of the BAPA-ase present in a total extract of barley proteins compared to the complete absence of this enzyme in the protein bodies. The pH optimum of the BAPA-ase is 8.6.

## Discussion

The results described here show that, while there appear to be some differences between the protein bodies of cereal grains, these are less than the differences between the cereals and the oilseeds. Protein bodies in barley, like those in wheat and rice, do not contain crystalloids or globoids but they do appear to have a fine structure of orderly, or lamellar, layers of some material(s) other than just storage protein. However, unlike those of wheat and rice, the fine structure of the barley particles, in many cases, appears to be an appendage (Fig. 3) which may be separable from the intact particle. This differs from the lamellar layers in wheat (6) and rice (8), which seem to occur entirely within the protein bodies. Some of the barley protein bodies do contain this fine structure within particles (insert, Fig. 2). The layers are often parallel (myelin-like structure) but can be observed in a grid-like pattern. The 2 patterns most likely represent layers in cross-section and in oblique or tangential view, suggesting that each layer is a 2-dimensional paracrystalline arrangement of materials.

The obvious question is, what is this fine structure and what, if any, is its function? If we compare this structure to the protein inclusions of salamander liver cells described by Fawcett (4) we see the same ordered layers of electron-dense and electron-transparent material. Both the liver tissue and these protein bodies were similarly fixed in phosphate-glutaraldehyde, osmium, and magnified to about the same dimensions for viewing. Thus, by analogy, we could expect the barley structures to contain layers of protein; but with something else sandwiched between. Jennings *et al.* (6) ascribe the lamellar structure of wheat protein bodies to a parallel array of lipoprotein membranes appressed to and surrounding the particles, which becomes an integral part of the protein bodies. We were unable to find similar areas of protein bodies surrounded by layers of lipoprotein membranes in intact barley aleurone, but this does not necessarily rule out that possibility.

Mitsuda *et al.* (8) isolated 3 fractions of protein bodies from rice, varying in lipid contents from 10 to 28 %. The fraction of higher density contained less lipid but higher carbohydrate. Their evidence showed that the stratified type of particles in rice were neither starch grains nor lipid particles but distinct protein bodies. When we extracted total lipids from the barley protein bodies, the total phosphorus content of the residue (table I) was increased slightly, suggesting that the amount of phospholipid phosphorus in the extracted lipids might be relatively small. Mitsuda *et al.* (9) found low lipid phosphorus in the rice protein bodies. They also found a small amount of RNA present but no values were reported. In 3 separate barley preparations reported here (table I) done in duplicate or triplicate, no RNA was found.

Mitsuda *et al.* (9) determined carbohydrate and protein (nitrogen × 6.0) and calculated lipid content as the difference from 100 %. Since protein contents in other cases have been estimated by multiplying nitrogen content by values of 6.0 to 7.0, we

Table I. *Composition of Barley Protein Bodies*

Protein bodies were prepared and analyzed as described in Materials and Methods. Values are averages of duplicate or triplicate determinations. Percentages are based on the dry weight of original material; phosphorus values represent $\mu$g/mg of protein bodies; nitrogen determination, done in triplicate, with st. dev. of 0.18 %.

| Sample | Nitrogen | Phosphorus | Lipid | RNA |
|---|---|---|---|---|
| | % | $\mu g/mg$ | % | % |
| Protein bodies, intact | 5.95 | 3.4 | 27 | 0 |
| Protein bodies, lipid-free | ... | 4.0 | ... | ... |

Table II. *Acid Phosphatase Activity of Protein Bodies on Different Substrates*

Preparation of protein bodies and the crude barley phosphatase, reaction conditions, and method of assay are described in Materials and Methods. Phosphate activity is listed as $10^{-4}$ $\mu$M $P_i$ hydrolyzed per min per quantity enzyme designated.

| Substrate | Activity measured: Protein bodies[1] | Crude barley[1] | Protein bodies[2] | Crude barley[2] |
|---|---|---|---|---|
| | $10^{-4}$ $\mu$M $P_i$ per min per test | | | |
| $\beta$-Glycerol-P | 0.87 | 11.5 | 0 | 14.9 |
| Sodium phytate | 58.0 | 8.1 | 6.6 | 9.6 |
| Glucose 1-P | 0 | 0 | 0 | 0 |
| Fructose 1,6-diP | 0 | 5.2 | 0 | 0.35 |
| ATP | 0 | 31.4 | 0 | 8.7 |

[1] Preparation 1, on weight basis; 2 mg enzyme source per test.

[2] Preparation 2, on nitrogen basis using a different preparation of protein bodies; 0.115 mg enzyme nitrogen per test.

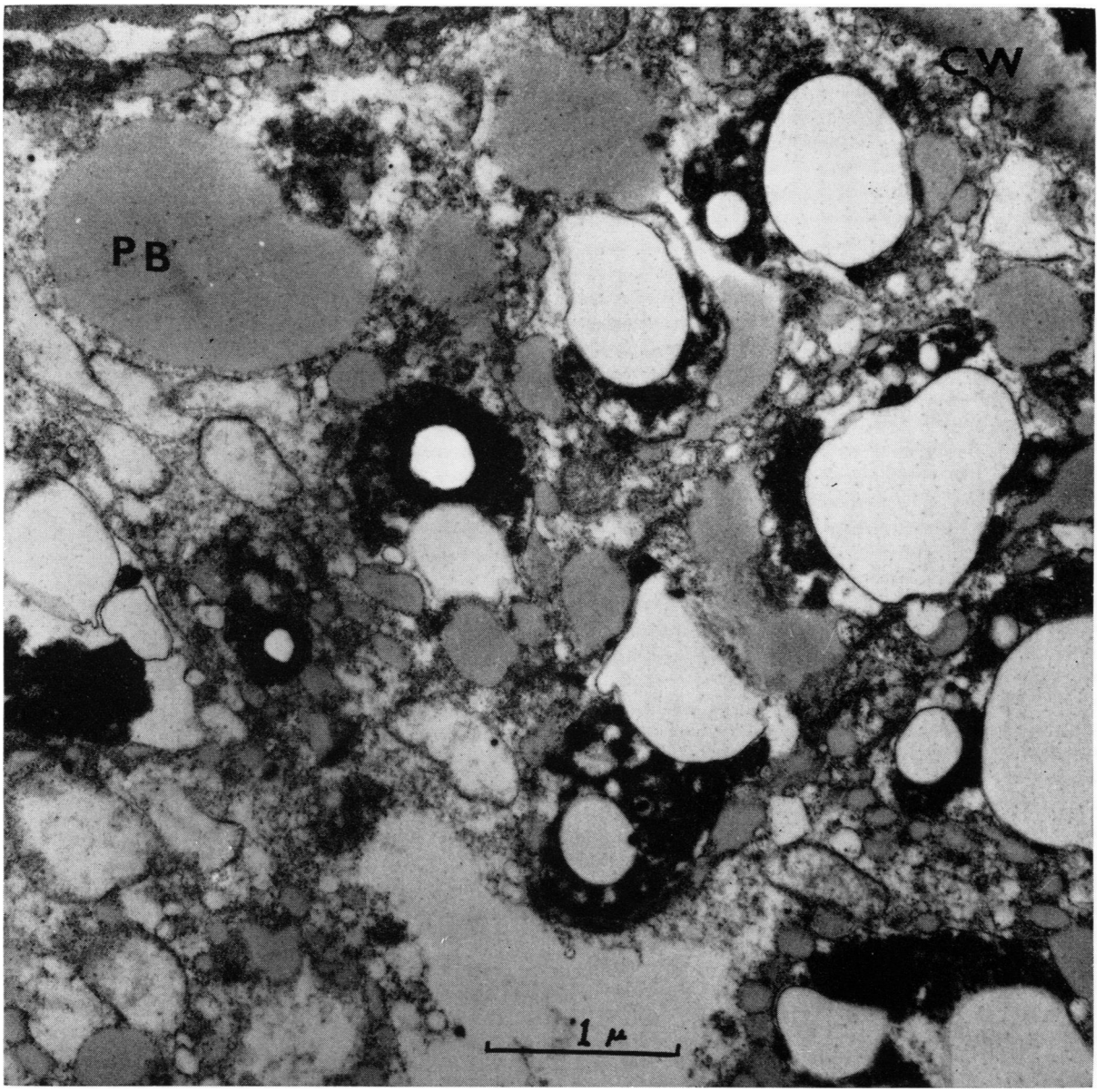


FIG. 1. Section through part of a cell from aleurone layer of dormant barley seed. Numerous protein bodies (PB) are found. The protein bodies are surrounded by a rough membrane. Large bodies surrounded by a complex membrane system including irregularly shaped electron-dense material are also evident. A part of the cell wall (CW) is shown. × 20,800.

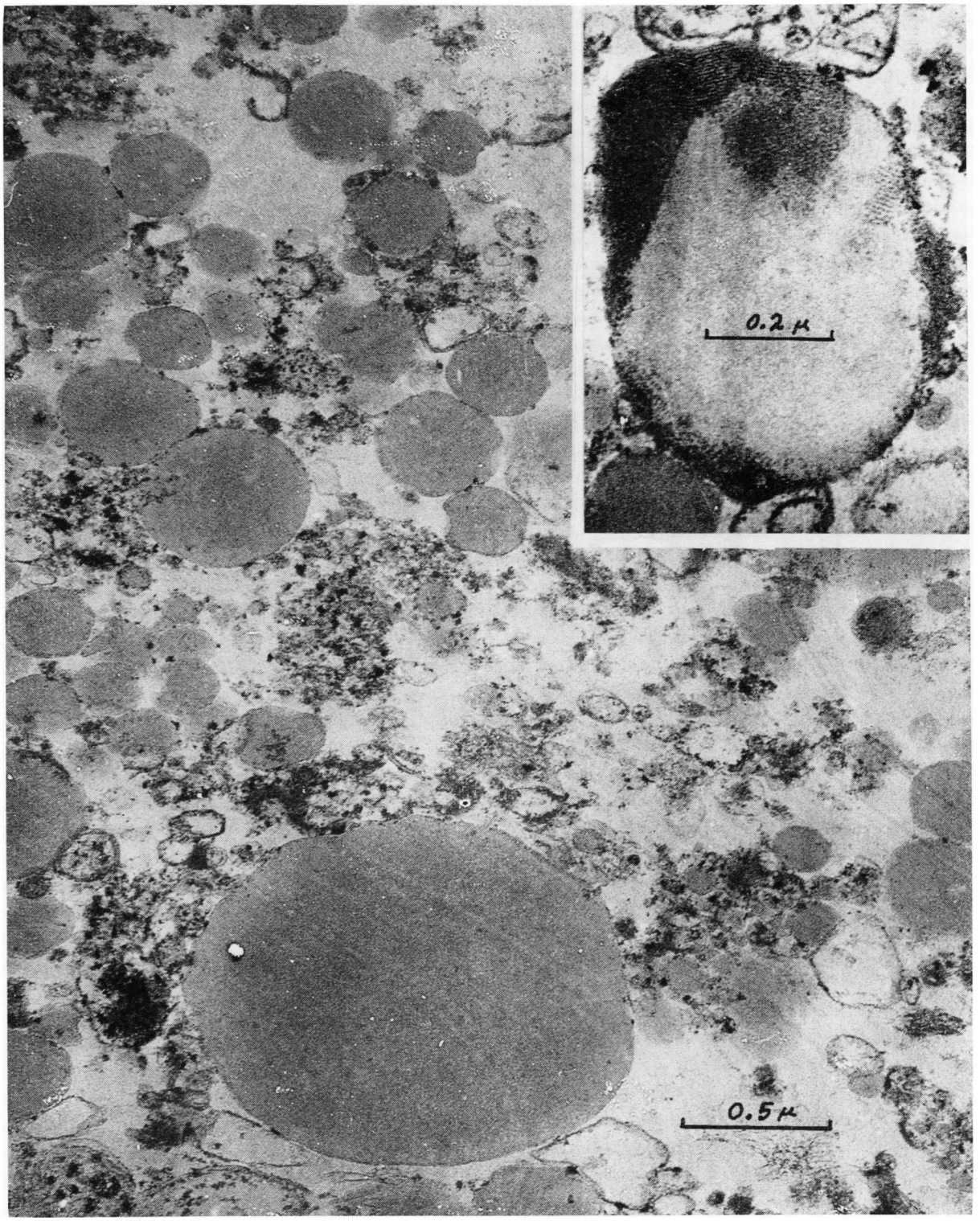


FIG. 2. Section through a preparation of protein bodies isolated from dormant barley seeds. Most of the protein bodies are surrounded by a single membrane. × 48,000. Insert: One of the larger protein bodies showing the layers of electron-dense material associated with the periphery of the particle. × 103,000.

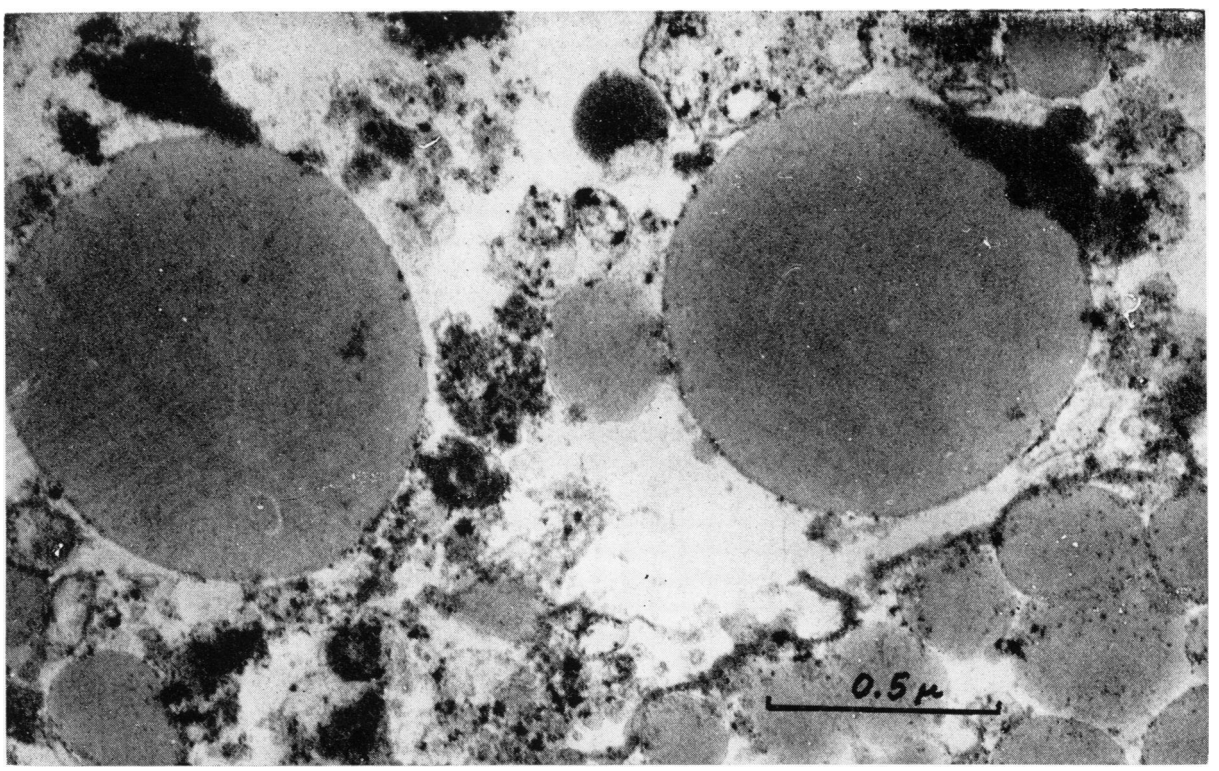


FIG. 3. Section through protein bodies isolated from dormant barley seeds. Attached to the large protein bodies are structures containing electron-dense material in ordered concentric layers. Upper left: the electron-dense material is partly detached from the protein body. × 61,000.

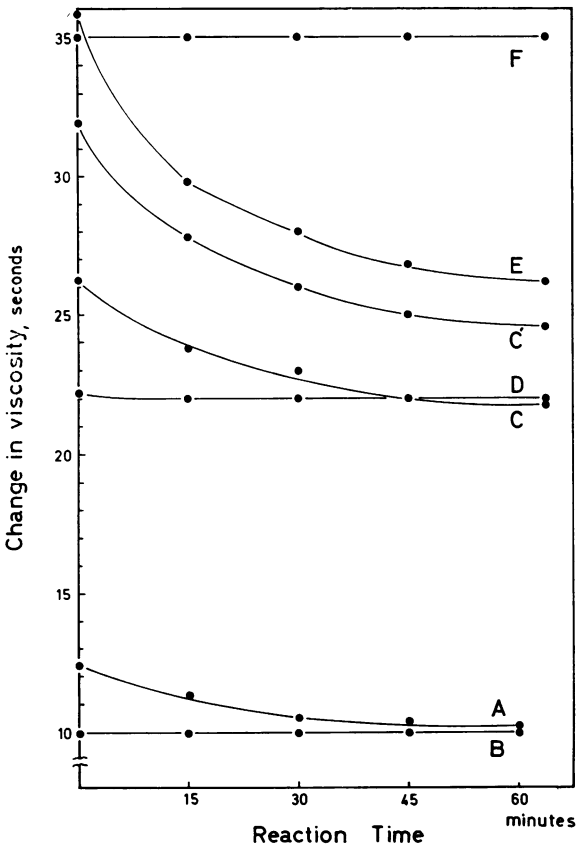


FIG. 4. Acid protease activity of protein bodies. Preparation of the protein bodies, reaction conditions, and method of assay are described in Materials and Methods. Activity is shown by a decrease in viscosity of a gelatin solution produced by enzyme hydrolysis. Curve A, 0.5 ml enzyme source (2 mg protein bodies) at pH 4.0; b, a control, same as A but boiled 15 min; C, 0.5 ml enzyme at pH 5.0; D, a control, same as C but boiled 15 min; $C^1$, 0.75 ml enzyme (3 mg protein bodies) at pH 5.0; E, 1.0 ml of enzyme (4 mg of protein bodies prepared without $\beta$-mercaptoethanol in buffer but added 18 hr before testing) at pH 5.0; F, 1.0 ml enzyme source as in E (before $\beta$-mercaptoethanol was added) at pH 5.0.

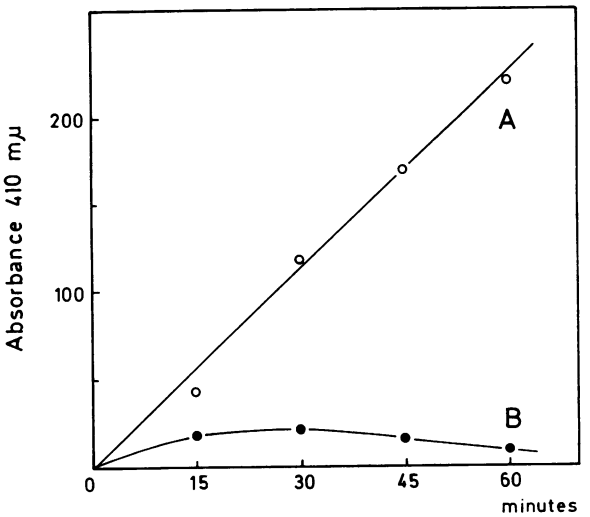


FIG. 5. BAPA-ase activity in barley seed extract compared to extracted protein bodies. Preparation of enzyme sources reaction conditions, and method of assay are described in Materials and Methods. A, barley seed; B, protein bodies.

list percent nitrogen only. Therefore the protein content of the barley protein bodies should be between 36 and 42 %. If we assume that the difference here (based on 100 %) is carbohydrate, then the barley protein bodies would contain 31 to 37 % carbohydrate. While this figure seems high we feel sure that this is not primarily contamination by starch grains. Very careful and extensive scanning of 3 different preparations in the electron microscope rarely showed free starch grains.

Another possibility is that these darker alternate layers in the fine structure might be the site of phytic acid storage. Phytic acid was found to bind readily to soybean protein (13). Since barley does not contain globoids such as the peanut (2) and cottonseed (7), this fine structure may be the counterpart of the oilseed globoids. Finding a specific phytase (table II) associated with these protein bodies tends to strengthen this hypothesis. Yatsu and Jacks (21) combining histochemistry with electron microscopy showed that the acid phosphatase of cottonseed protein bodies was distributed throughout the particle. In the castor bean (11), the acid lipase was shown to be associated with the spherosome membrane. However, the present results on the barley phytase do not permit a further subcellular localization of the enzyme within or on the particle.

Though 5 different proteases have been separated from dormant barley seeds (3) the absence of the BAPA-ase, which acts at pH 8.6, suggests that only acid hydrolase activity may be associated with the protein bodies. This would be similar to the systems existing in cottonseed (21) in which acid phosphatase and acid proteinase activity were found in the aleurone grains. The results in Fig. 4 suggest that the initial production of amino acids from barley reserve proteins upon germination is catalyzed by an acid protease. Whether this is mediated by one or more of the 4 acid proteases reported (3) cannot be ascertained here. However, the requirement for sulfhydryl reagents to maintain proteolytic activity in the particles and the restoration of activity of an inactive enzyme by incubation with sulfhydryl reagents (Fig. 4, curve E) suggests that the SH-group of the protease(s) in the protein body is quite labile.

If the protein bodies with fine structure do indeed have some particular biological function, in addition to being reservoirs for storage proteins, their concentration in specific areas of the aleurone layer or in the embryo of the barley seed may shed some

light on possible reasons for the 2 different types of protein bodies. Characterization of the fine structure and subcellular localization of the phytase and possibly other enzymes associated with the protein bodies, depends upon the separation of this structure from the storage protein contained therein. Work towards these goals is continuing and will be reported later.

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