Cereal Chemistry

BIOCHEMICAL PROPERTIES AND ULTRASTRUCTURE OF PROTEIN BODIES ISOLATED FROM SELECTED CEREALS

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ABSTRACT   Cereal Chemistry 53(1): 1-12

The protein bodies of millet (*Eleusine coracana* Gaertn.), barley (*Hordeum vulgare* L.), babala (*Pennisetum typhoideum* Burm.), white maize (*Zea mays* L.), and sorghum *(Sorghum bicolor* (L.) Moench), isolated by a new method, are distinct organelles differing markedly in their ultrastructure. Wheat *(Triticum aestivum* L.) lacks identifiable protein bodies although a protein-rich fraction was obtained. The protein bodies and the wheat protein preparation are composed of predominantly insoluble protein and have insoluble protease activity. Phytase activity is more variable being undetectable in wheat and millet but present in the other cereal preparations. The products obtained varied from 51% protein in wheat to 79% protein in maize and thus the method used yields protein-rich fractions from different cereals in a relatively simple and rapid process.

Protein bodies have been observed in several mature cereals with the exception of wheat (1), although they are found in the endosperm of developing wheat (2). In most cereals, protein bodies seem to be embedded in an amorphous matrix of protein and this complex is tightly packed around the starch granules. Disruption of this protein-starch mixture in mature grain is difficult and this has inhibited detailed studies of protein bodies in mature grain. Consequently, most ultrastructural studies on cereal protein bodies have been carried out on sections of cereal grain (3,4). Where protein bodies have been isolated, usually immature grain was used (2,5).

Protein bodies from barley aleurone tissue have associated acid protease, acid phosphatase, and β-amylase activities (6,7). Isolated sorghum protein bodies have acid protease, acid phosphatase, glycosidase, and nuclease activities (8). Rost (9), however, was unable to detect acid phosphatase in sections of protein bodies from *Setaria lutescens*.

The ultrastructure of protein bodies in cereals has received more attention than biochemical studies. Barley aleurone tissue protein bodies were of two types (6): one was composed of a homogeneous material; the other type had a layered structure. Bands staining with different intensities, also suggesting a layered structure, were observed in immature protein bodies of maize (10), although

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mature granules were more homogeneous. Protein bodies in sections of mature seeds of *S. lutescens* (9), rice (11), and proso millet (12) all had an obvious layered internal structure.

A somewhat similar pattern has been observed in sorghum protein bodies *in situ* (3) and in isolated sorghum protein bodies (13). It appears that protein bodies, in a variety of seeds, have a layered internal structure suggesting heterogeneity. Although mature wheat apparently has no protein bodies, purified wheat protein preparations have a variety of inclusions in a matrix (14).

In an attempt to provide more information on the nature of cereal proteins, we have isolated protein bodies from mature seeds of millet, barley, babala, maize, and sorghum and a protein preparation from wheat. Some biochemical properties and the ultrastructure of these materials have been studied.

MATERIALS AND METHODS

Grain Preparation

Air-dried samples of viable seeds of millet (*Eleusine coracana* Gaertn.), barley *(Hordeum vulgare* L.), babala (*Pennisetum typhoideum* Burm.), white maize *(Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Moench), and wheat (*Triticum aestivum* L.) were milled through a 1-mm sieve on an EBC mill (Casella Company, London). The cereal meal was then defatted in acetone at room temperature with a grain to acetone ratio of 1:2. The defatted powder was air-dried and kept refrigerated until used.

Preparation of Protein Bodies

The same procedure was followed with all cereals. Acetone powder was suspended in 1% (w/v) sodium metabisulfite solution with a powder to solution ratio of 1:5 and stirred for 30 min at room temperature. The slurry was passed twice through a Fryma mill (Leumann and Uhlmann, West Germany), and then through a nylon cloth. The filtrate from the cloth was pumped through a Sharple's super centrifuge operating at low speed with a flow rate of 900 ml/min. Starch granules were retained in the barrel of the centrifuge and were discarded. The supernatant liquid was pumped once again through the centrifuge operating at high speed with a flow rate of 300 ml/min. Protein bodies were deposited in the barrel of the centrifuge. These were washed with water and either dehydrated in acetone for chemical analysis or resuspended in $0.1 M$ citrate buffer, pH 6.2, for determination of enzyme activities and for electron microscopy.

Chemical Analysis

Total nitrogen and phosphorus were determined on oven-dried samples of acetone-dehydrated protein bodies by the procedure of Thomas *et al.* (15). Metals were estimated by atomic absorption spectroscopy on the same digests used for nitrogen and phosphorus analysis.

Enzyme Assays and Protein Determination

Aliquots (20 ml) of the suspension of protein bodies in $0.1 M$ citrate, pH 6.2, were centrifuged at $27,000 \times g$ for 10 min and the supernatant discarded. The sedimented protein bodies were ground in a mortar and pestle in 20 ml of fresh $0.1 M$ citrate buffer, pH 6.2, and then centrifuged at $27,000 \times g$ for 10 min. The

supernatant was retained and assayed for soluble enzyme activity and soluble protein. The extracted protein bodies were resuspended in 20 ml of $0.1 M$ citrate buffer, pH 6.2, and assayed for insoluble enzyme activity and insoluble protein.

In a final volume of 1.5 ml, the protease assay mixture contained 250 μmol of citrate buffer, pH 4.6; 2.5 mg hemoglobin; and 0.5 ml enzyme. The mixture was incubated for 30 min at 55° C and the reaction stopped by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. Denatured protein was sedimented by centrifugation at $2,300 \times g$ for 10 min and the free amino nitrogen in the supernatant determined by the method of Fields (16). A unit of activity is defined as a μmol of glycine equivalent released per hour.

In a final volume of 1.5 ml, the phytase assay mixture contained 100 μmol of acetate buffer, pH 5.0; 2.0 μmol of sodium phytate; and 0.5 ml enzyme. After incubation for 1 hr at 40° C, the reaction was stopped by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. Denatured protein was sedimented by centrifugation and inorganic phosphate in the supernatant determined as described by Barrett (17). A unit of activity is defined as a μmol of inorganic phosphate released per hour.

Soluble and insoluble protein in the extracts was determined by alkaline hydrolysis and ninhydrin determination as described by McGrath (18).

Preparation of Samples for Electron Microscopy

Aliquots (10 ml) of a suspension of protein bodies in $0.1 M$ citrate buffer, pH 6.2, were centrifuged at $27,000 \times g$ for 10 min and the supernatant discarded. The sedimented protein bodies were dispersed in 5% glutaraldehyde in $0.05 M$ phosphate buffer, pH 7.2, and left for 1 hr in ice. The suspension was then centrifuged at $27,000 \times g$ for 10 min, the glutaraldehyde removed, and the protein bodies resuspended in a small amount of agar at 50° C. When the agar solidified, the block was chopped into small pieces and stored in $0.05 M$ phosphate buffer, pH 7.0. The agar blocks were then post-fixed in 1% osmium tetroxide in $0.05 M$ phosphate buffer, pH 7.3. After dehydration in ethanol and propylene oxide, the specimens were embedded in an epon/araldite mixture. Samples were sectioned with a diamond knife and double-stained with uranyl acetate and lead citrate.

RESULTS AND DISCUSSION

Protein body preparations from all cereals were considerably enriched in nitrogen compared to whole grain (Table I). With the conventional conversion factor of 6.25, the protein bodies vary from about 79% protein in maize to about 51% protein for the wheat preparation. The phosphorus content is quite low in all protein body preparations, suggesting they are not major phosphorus storing organelles. Protein bodies prepared from barley aleurone tissue were previously shown to consist of 5.95% nitrogen and 0.34% phosphorus (6), which is considerably lower than the barley protein bodies isolated here from whole grain. All the preparations stained intensely with acid fast green (4), confirming their proteinaceous character.

The metal content of the protein bodies was uniformly low and, in most cases, much lower than in the original grain (Table I). This suggests that these organelles are not repositories for metals in the resting grains.

Examination of millet, babala, maize, and sorghum protein body preparations

under the light microscope showed very few starch granules and the protein bodies did not stain with iodine. Barley and wheat preparations were contaminated with small starch granules. In all cases, however, the starch granules were clearly separated from the protein.

Proteolytic activity against hemoglobin was manifested by all the preparations (Table II). Furthermore, this activity was predominantly insoluble in aqueous buffer. Phytase activity was more variable, being undetectable in wheat and millet preparations (Table II). Sorghum and babala had low levels of insoluble activities with no detectable soluble activity. Barley and maize, however, had significant levels of both soluble and insoluble phytase. There is little information on the enzymatic characteristics of cereal protein bodies, although Ory and Henningsen (6) have shown that protein bodies from barley aleurone layer have both phytase and acid protease activities which is in agreement with the data shown here (Table II). Sorghum protein bodies contain, in addition to protease and phytase, α-glucosidase, β-glucosidase, β-galactosidase, and several acid phosphatases (8). Again these activities were all predominantly insoluble. Acid phosphatase could not be detected in protein bodies of *S. lutescens* (9) and there may be greater variation in acid phosphatase distribution than in protease distribution.

The majority of the protein in all protein body preparations is insoluble in aqueous buffer (Table II). However, a greater proportion of the total enzymatic activity is soluble than of the total protein. This suggests that either the enzymes are solubilized more readily than the bulk protein, or that they are surface contaminants of the protein bodies. However, the extensive aqueous treatments involved in preparation of the protein bodies still leave significant activity in an insoluble form which is consistent with the activity being an integral part of the particles. Alternatively, the enzymes could be associated with nonprotein body material, although we have no evidence that this is the case.

Protein bodies of millet appear as discrete organelles possibly surrounded by a membrane (Fig. 1). There is a slight suggestion of an internal structure as some

TABLE I
Chemical Composition of Whole Grains and Protein Bodies of Selected Cereals[a]

		Component Analyzed				
Cereal	Sample Analyzed	N	P	Ca	K	Mg
Millet	Whole grain	1.11	0.22	0.06	0.58	0.17
	Protein bodies	10.16	0.43	0.09	0.06	0.06
Barley	Whole grain	1.40	0.43	0.03	0.56	0.16
	Protein bodies	8.61	0.62	0.09	0.08	0.06
Babala	Whole grain	1.58	0.33	0.01	0.46	0.14
	Protein bodies	11.39	0.69	0.01	0.09	0.07
Maize	Whole grain	1.40	0.26	0.01	0.36	0.13
	Protein bodies	12.58	0.50	0.01	0.03	0.01
Sorghum	Whole grain	1.24	0.33	0.01	0.40	0.15
	Protein bodies	10.95	0.37	0.02	0.02	0.01
Wheat	Whole grain	2.44	0.21	0.04	0.43	0.13
	Protein preparation	8.10	0.31	0.03	0.03	0.01

[a]Values expressed as per cent of oven dry weight of defatted grain or of acetone dehydrated protein bodies.

granules have a darker-staining central area. A similar arrangement was observed in proso millet (12). Many granules, however, appear relatively homogeneous in section. They vary in diameter from less than 1 μm to about 2 μm which again is a similar size range to those granules studied *in situ* in proso millet (12).

Barley protein bodies are somewhat similar to those isolated by Ory and Henningsen (6). There is little evidence of any internal structure, but there appears to be associated fine structure around the bodies (Fig. 2). The peripheries of the granules are not sharp as in millet, but more diffuse and do not appear to be membrane bounded. The granules are rather elliptical in section with a minimum diameter of about 1 μm and a longitudinal diameter of up to 2 μm.

Protein bodies of babala have quite a different appearance (Figs. 3 and 4). They have discrete edges, possibly surrounded by a membrane. These granules have a very obvious internal structure with numerous spots of darker-staining material arranged in concentric rings. A similar internal structure has been observed in rice protein bodies (11). Babala protein bodies are also a different shape from those of other cereals. They seem to have invaginations and protuberances which may be the result of fusion and deformation of adjacent granules. The babala preparation shows frequent clumps which seem to be made up of granules fused together (Figs. 3 and 4). They are larger than those from other cereals ranging in cross-section from about 1.5 to 4 μm.

Maize protein bodies are fairly circular in section and some of the smaller ones are homogeneous in composition (Fig. 5). There is always a considerable amount of fine structure adhering to the outside edge of the granules, somewhat similar to that seen in barley (Fig. 2). There is evidence of a membrane along the periphery of these granules in places. A considerable number of the protein bodies have vacuoles arranged in an apparently random manner (Figs. 5 and 6). Within these vacuoles there are often seen intensely staining aggregations. The nature of this material is unknown, although its apparently specific location in the vacuoles of

TABLE II
Distribution of Protease, Phytase, and Protein in
Protein Body Preparations of Selected Cereals

Cereal	Fraction	Protease[a]	Phytase[b]	Protein[c]
Millet	Soluble	1.72	0	0.96
	Insoluble	3.04	0	12.88
Barley	Soluble	1.40	0.30	1.94
	Insoluble	4.60	0.21	21.68
Babala	Soluble	2.68	0	3.16
	Insoluble	5.12	0.12	11.56
Maize	Soluble	1.44	0.17	1.46
	Insoluble	2.80	0.30	13.20
Sorghum	Soluble	0.38	0	0.30
	Insoluble	2.72	0.10	10.90
Wheat	Soluble	2.68	0	2.58
	Insoluble	2.88	0	14.88

[a]Protease activity: μmol glycine equivalent released/hr/ml.
[b]Phytase activity: μmol P_i released/hr/ml.
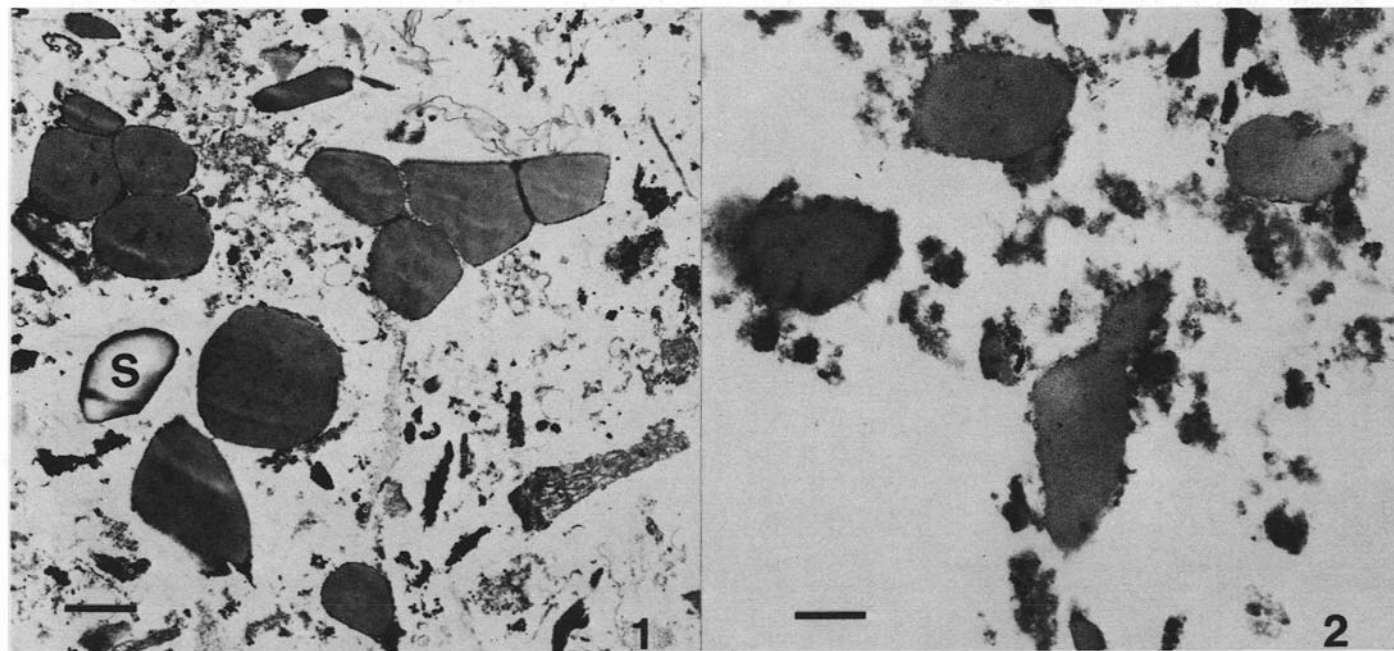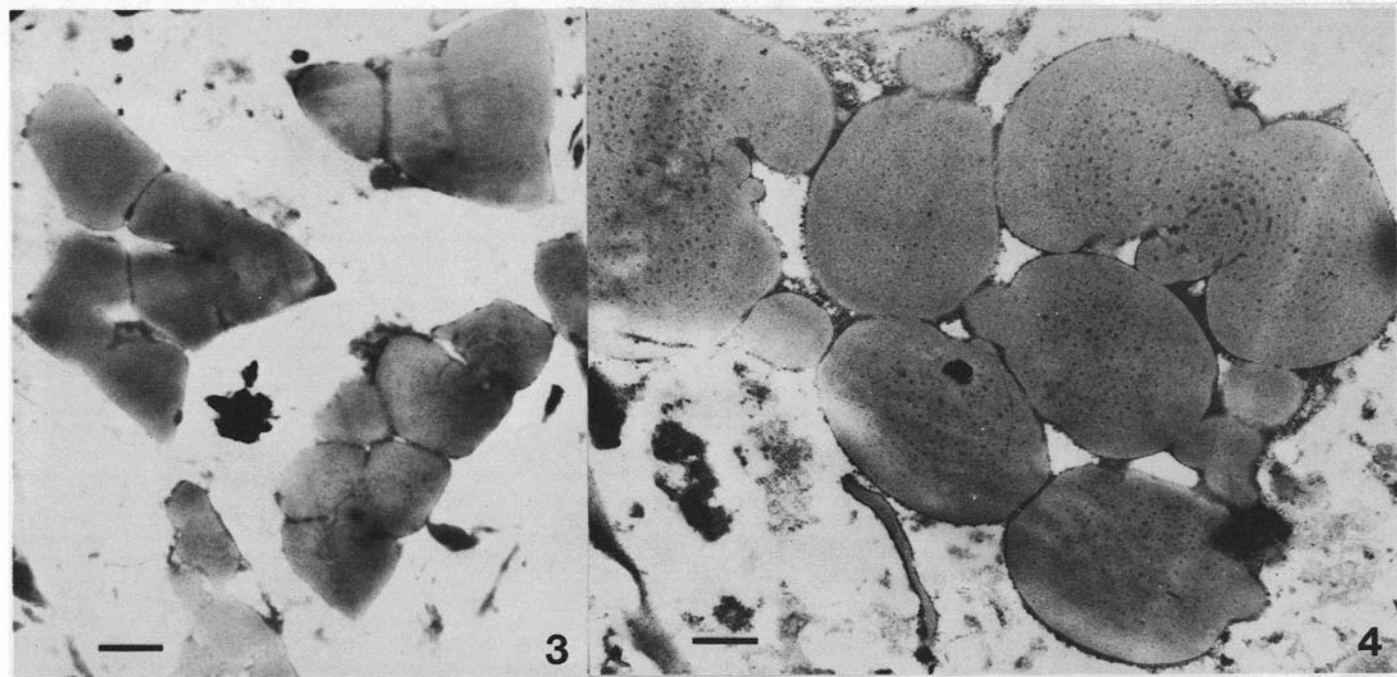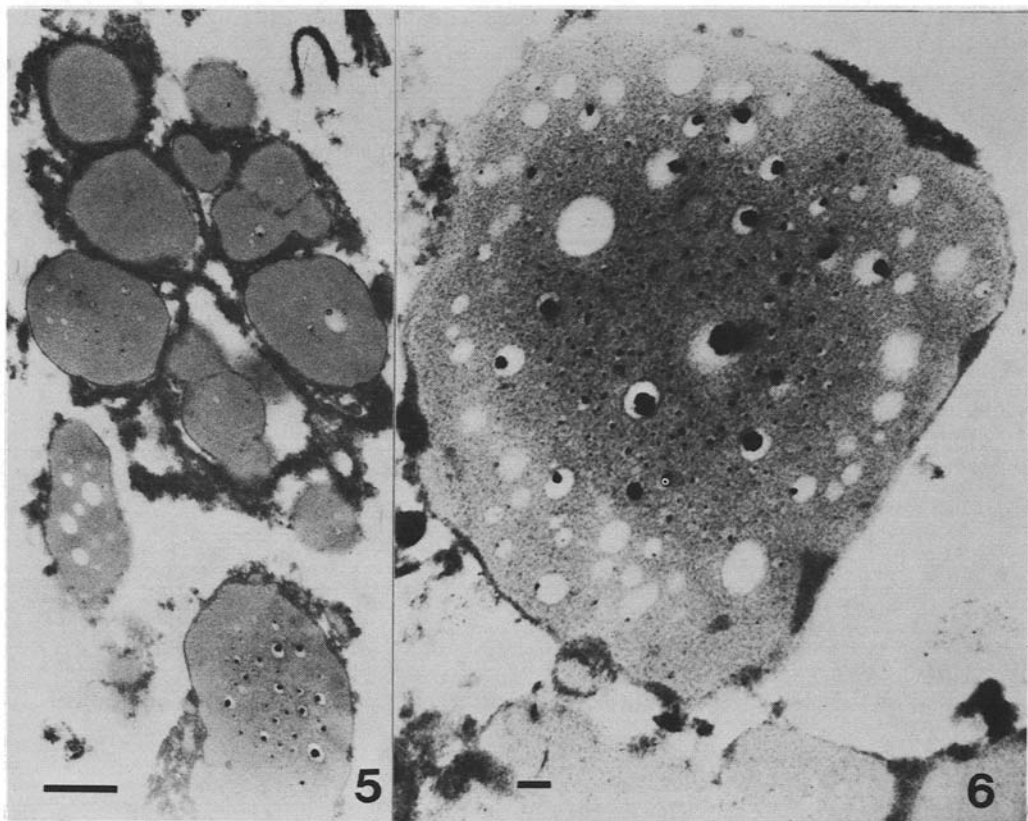[c]Protein concentration: mg/ml.

Fig. 1. Protein bodies isolated from millet, with a contaminating starch granule (S). Scale line represents 1 μm. Fig. 2. Protein bodies isolated from barley. Scale line represents 0.5 μm.

Figs. 3 and 4. Protein bodies isolated from babala. Scale line in Fig. 3 represents 0.5 μm and in Fig. 4 represents 1 μm.

Figs. 5 and 6. Protein bodies isolated from maize. Scale line in Fig. 5 represents 0.5 μm and in Fig. 6 represents 0.1 μm.
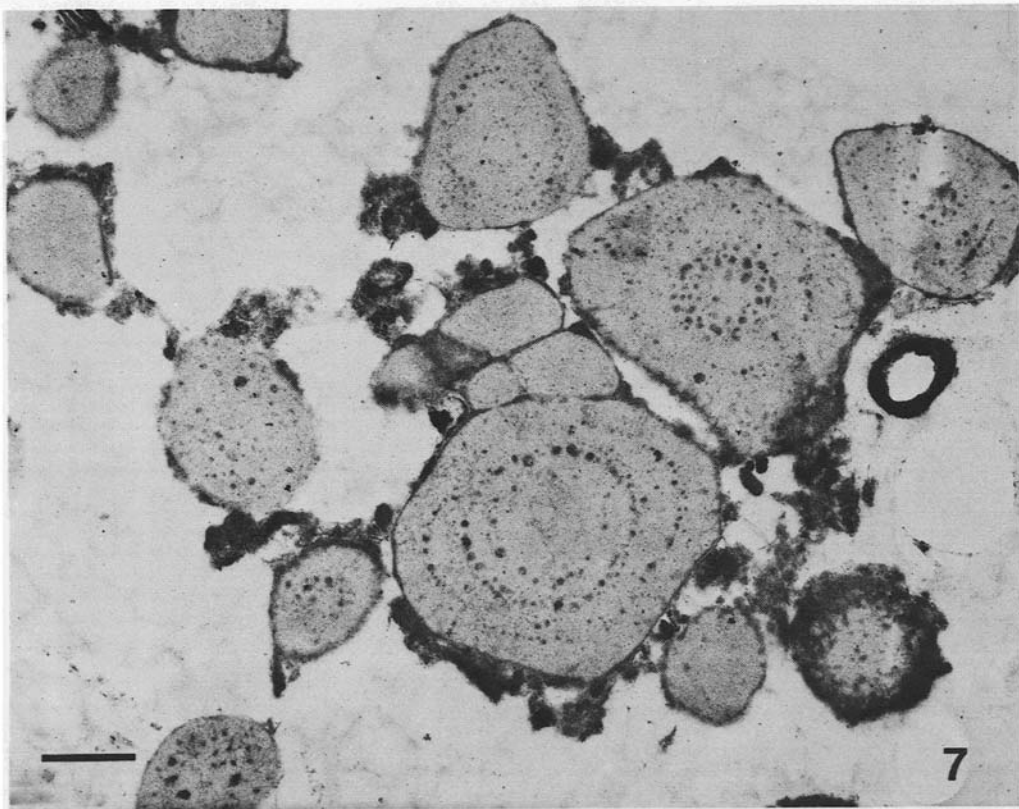
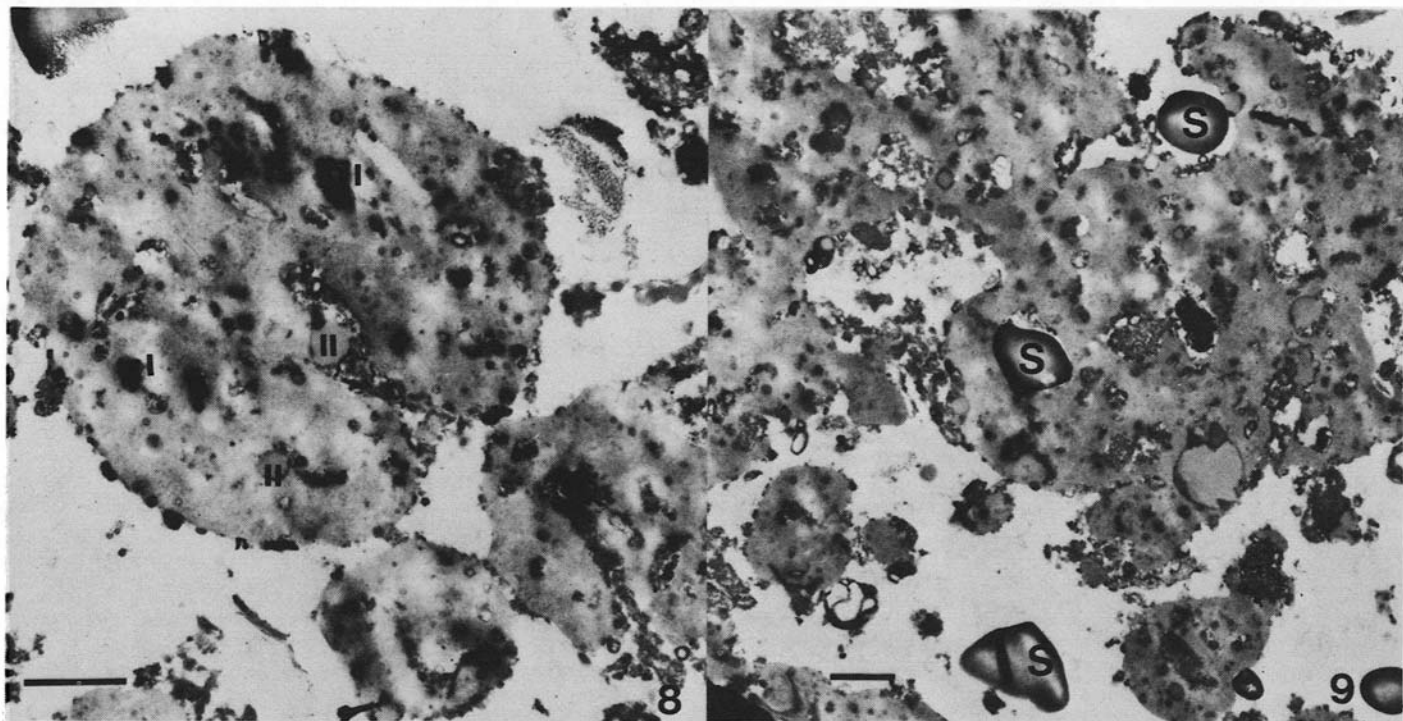Fig. 7. Protein bodies isolated from sorghum. Scale line represents 1 μm.

Fig. 8. Protein preparation from wheat showing circular section with type I and type II inclusions. Scale line represents 1 μm. Fig. 9. Protein preparation from wheat showing undefined mass of protein with contaminating starch granules (S). Scale line represents 1 μm.

the protein bodies suggests it is not an artifact. Higher power magnification of vacuolated maize protein bodies (Fig. 6) shows that there is a large number of vacuoles varying in size within the protein granules. The granules have diameters between 1 and 2 μm with the vacuoles as large as 0.2 μm in diameter. None of the other cereals studied has vacuolated protein bodies.

Sorghum protein bodies (Fig. 7) are mostly circular in section and vary in size from 0.5 to 3.5 μm. Some of the granules are in close juxtaposition with neighboring ones, but still have a distinct border separating them, unlike babala, where the protein bodies often appear to be fused together. The granules of sorghum have a discrete border which may be membrane bounded. These isolated protein bodies are quite similar to those observed *in situ* by Seckinger and Wolf (3). Furthermore, both isolated and *in situ* protein bodies show a considerable amount of internal structure having darker-staining material arranged in concentric rings.

Wheat preparations have a very different appearance. The samples contain large masses of material often arranged in circular sections (Fig. 8), which appear to have a limiting border and vary in diameter from about 2.5 to 5.0 μm. Other wheat preparations appear as a rather undefined mass (Fig. 9). In both cases, however, the structure seems to be similar, consisting of a matrix in which various inclusions are visible. Some of these are similar to the type I and II inclusions described by Simmonds (19) in wheat dough. Type I inclusions are irregular in outline and intensely staining. Simmonds (19) suggests that they may be derived from endoplasmic reticulum. Type II inclusions are circular in section and may be lipid-rich droplets. Figure 9 also shows that the contaminating starch granules are clearly separated from the protein matrix.

CONCLUSIONS

Five of the six cereals studied here—millet, barley, babala, maize, and sorghum—have distinct protein bodies. The analytical and histochemical data suggest that these cereal protein bodies are primarily composed of storage protein and enzymes, having little phosphorus or metals. The protein bodies prepared here are clearly separated from the starch granules. The close similarity in ultrastructure between isolated sorghum protein bodies and those observed *in situ* (3) suggest that the protein bodies are obtained essentially unchanged from their natural state.

Wheat appears to be lacking in identifiable protein bodies, although the method used here produced a protein-rich fraction. The presence of protein bodies in immature wheat endosperm has been reported (2). However, at maturity, these have disappeared and are replaced by a matrix type of protein. Wheat appears to have evolved a form of protein storage which is largely amorphous material. Other cereals have evolved a protein storage system of protein granules in a small amount of amorphous protein matrix. This is consistent with the fact that, of the cereals considered here, only wheat is suitable for breadmaking.

Despite the ultrastructural differences, there is little variation in protease activity and protein solubility of the protein bodies and wheat protein preparation. Proteolytic activity associated with insoluble protein seems a common feature of

cereal grains. Protein bodies also contain several other enzymes (6,7,8). Therefore, cereal protein bodies may be storage forms of enzymes as well as reserve protein.

The method described here for preparation of protein bodies successfully disrupts the starch-protein complex of mature cereal grains. This allows the production of a protein-rich fraction from all the cereals studied in a relatively simple and rapid process. Therefore, this method may have general applications in cereal technology as a means of producing protein-rich materials from cereal grains.

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