SUBCELLULAR DISTRIBUTION AND ENZYME DIGESTIBILITY OF ENDOSPERM PROTEINS OF AMYLOMAIZE AND NORMAL CORN

M. J. WOLF and U. KHOO, Northern Regional Research Laboratory[1], Peoria, IL 61604

ABSTRACT

Endosperm storage protein of amylomaize contains protein bodies averaging about 1.16 μm in diameter as compared with an average of 1.50 μm for normal dent corn. Both alcohol-soluble and alcohol-insoluble proteins are dispersed throughout the protein bodies of amylomaize. In normal corn, alcohol-insoluble protein is condensed in the central part of the protein body and zein in the peripheral. The protein bodies of amylomaize appear to contain a larger proportion of alcohol-insoluble protein than those of normal dent corn. In addition to protein bodies, numerous smaller structures, about 0.2 μm in diameter, are embedded in the matrix protein of both amylomaize and normal corn. In both types of corn, Pronase digests both matrix and zein body proteins in thin sections where contents of the bodies are exposed to the enzyme solution. Under these conditions the matrix proteins are more rapidly digested than the proteins of the bodies.

The amylose extender gene (*ae*), which markedly increases the iodine sorptive capacity of corn endosperm starch, also affects the total protein content (1) and composition of endosperm proteins. Baudet *et al.* (2) reported that the lysine content of amylomaize mutants was intermediate between that of normal maize and *opaque-2*. We found (3) that endosperm protein of commercial amylomaize hybrids was significantly higher in lysine and other amino acids than normal dent hybrids, but amino acid content of a high-amylose inbred line was similar to that of normal corn. Differences in endosperm protein composition may be accompanied by corresponding microscopic modifications of protein structural features at the subcellular level. Consequently, one objective of the work reported here is to compare the subcellular distribution of the endosperm storage proteins of amylomaize with that of ordinary corn to identify changes influenced by the *ae* gene. In addition, we studied digestibility and solubility of subcellular protein structures as a basis for evaluating quality of amylomaize endosperm proteins relative to that of ordinary corn.

MATERIALS AND METHODS

The inbred variety W64A and the corresponding line W64A*ae* homozygous for the *ae* gene were studied. This near-isogenic pair, differing only in the single gene *ae*, was produced by M. S. Zuber at Columbia, Mo. Amylose contents of the respective starches were W64A, 32%, and W64A*ae*, 61%. In addition, two single-cross hybrids, both from Bear Hybrid Corn Co. of Decatur, Ill., were examined. One of these was a normal dent hybrid which served as a control and the other was a high-amylose line designated as "class 8." Amylose content of the starches of these hybrids was 28 and 81%, respectively. Detailed data on these samples, including analyses of the endosperm proteins, will be found in (3).

[1]Agricultural Research Service, U.S. Department of Agriculture.

Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Copyright © 1975 American Association of Cereal Chemists, Inc., 3340 Pilot Knob Road, St. Paul, Minnesota 55121. All rights reserved.

Electron Microscopy

A rapid, greatly simplified preparative procedure was developed in which the usual fixation and infiltration of tissue were omitted. Untreated endosperm fragments, taken from the outer endosperm, were merely embedded in low viscosity resin (4) for support and sectioned at 0.15 to 0.20 μm with a glass or diamond knife. The sections were fixed in 4% formaldehyde solution and mounted on copper grids coated with collodion. Further fixing or staining with 1% uranyl acetate (5 to 10 min), 1% osmic acid in 0.1 *M* pH 7.1 phosphate buffer (15 min), or lead citrate (5) was accomplished on the grid-mounted sections. Sections on the grids were treated with 0.5% Pronase in 0.1 *M* pH 8.0 phosphate buffer for 30 min to 2 hr to digest proteins in the section. In some cases, preliminary removal of part or all of the starch from the sections facilitated observation of protein structures. Sections on the grids were destarched by treatment with salivary α-amylase in 0.05 *M* pH 6.8 phosphate buffer for 10 min. All digestions were carried out at room temperature.

Alcohol-soluble protein, zein, was extracted from the grid-mounted sections by treating for 45 min with 70% ethanol at room temperature. Longer treatment with alcohol produced no further change in the sections. Cutting sections at a thickness of 0.15 to 0.20 μm ensured direct exposure of protein body contents to solvents, enzyme solutions, fixatives, and staining reagents.

Measurements of protein body diameters were made from electron micrographs.

RESULTS

Protein bodies of amylomaize are smaller than those of ordinary corn. The average diameter of protein bodies of W64A*ae* was 1.11 μm as against 1.57 μm in the normal counterpart W64A. In class 8 amylomaize, the protein bodies averaged 1.18 μm in diameter *vs.* 1.50 μm in the normal dent hybrid. Differences in the mean diameters are significant at the 1% level in the near-isogenic pair and at the 5% level in the amylomaize hybrid *vs.* the normal dent hybrid.

Previous work showed that combining histochemical information as to the pattern of protein distribution with the pattern of alcohol solubility served as a means of identifying sites occupied by zein and nonzein protein in maize endosperm (6,7). We adhered to this plan of differentiating proteins at the subcellular level in the work which follows.

Effect of Pronase Digestion

Treatment of the sections with Pronase resulted in stepwise digestion of the storage proteins filling in between the starch granules. In the first stage, the matrix protein surrounding the bodies was digested (Fig. 1a), followed by solubilization of the protein body contents after additional incubation (Fig. 1b). Small amounts of undigested residue of the storage proteins appeared to be made up of membranes and possibly some fine, granular material, both apparently derived from the protein bodies (Fig. 1b). Thus, treatment with Pronase demonstrated that the material between the starch granules, including both the bodies and the surrounding matrix, was made up largely of proteins.

Rate of protein digestion was variable. Matrix protein was digested in sections in 30 to 60 min; zein body protein was solubilized in 1 to 2 hr. The pattern of

digestion of matrix and zein body proteins of all varieties studied followed the same order as that of class 8 amylomaize shown in Figs. 1a and 1b.

Protein bodies briefly treated with Pronase were heavily stained by electron stains (Fig. 1a). As discussed below, electron stains have little effect on untreated protein bodies.

Effect of Ethanol Treatment

The pattern of extraction of endosperm proteins of the various corns when treated with ethanol is compared with the corresponding untreated protein in Figs. 2 through 6. Protein bodies of untreated sections in both ordinary and high-amylose corn are remarkable for their low affinity for electron stains (8). No combination of fixative and stain adequately stained untreated protein bodies. In sections not treated with ethanol, a pattern of moderately electron-dense material may be faintly discernible in the bodies, particularly in normal corn (Figs. 2a, 3a, 4a, and 6a), but most of the body appears grayish. The surrounding heavily stained, amorphous matrix protein stands out in sharp contrast. Ethanol treatment had no apparent effect on the matrix protein.

Normal Corn

Protein bodies of normal corn, including those of both the dent hybrid and the inbred W64A, when modified by alcohol extraction show a clear peripheral area and a deeply stained central core (Figs. 2b and 3b). This pattern demonstrates that the prolamine, zein, is localized in the peripheral part of the protein body in normal corn. The alcohol-insoluble central mass, digestible by Pronase,

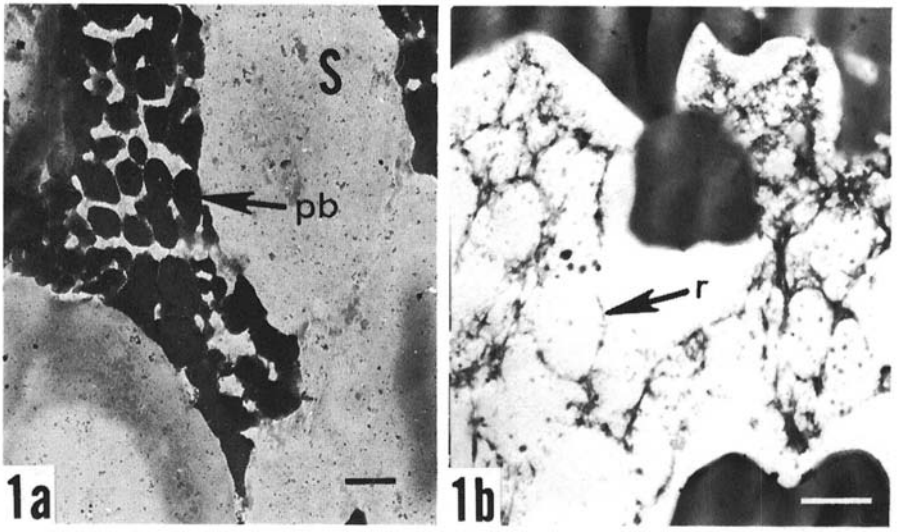

Fig. 1. Endosperm of amylomaize, class 8. Osmium tetroxide. (a) Free protein bodies after solubilizing the matrix protein with Pronase for 30 min (×6800); (b) residue left after solubilizing both matrix protein and protein body contents with Pronase for 60 min (×9900).

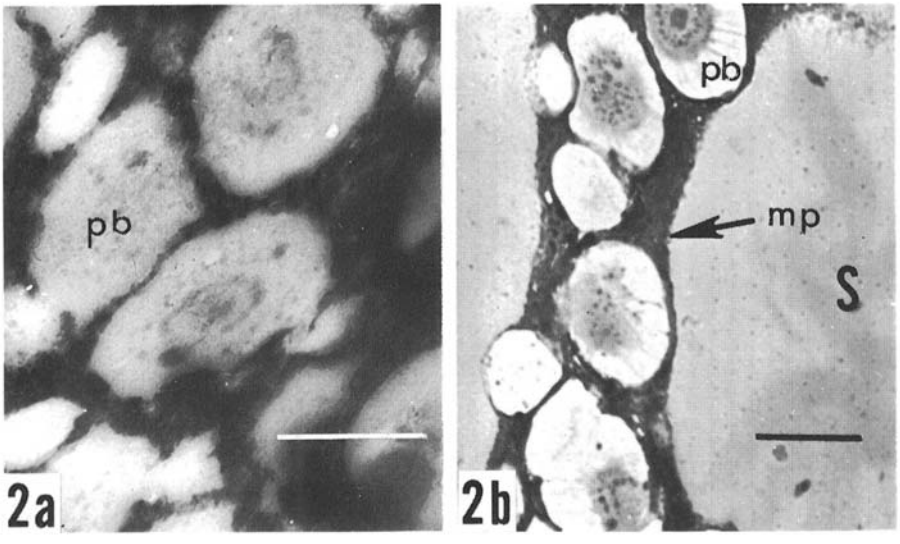

Fig. 2. Endosperm of normal hybrid corn. Formaldehyde and uranyl acetate. (a) Untreated protein (×20,000); (b) ethanol-extracted protein bodies (×14,000).

Line scale = 1 μm; S = starch granules; pb = protein body; mp = matrix protein; r = membranous residue after Pronase digestion.

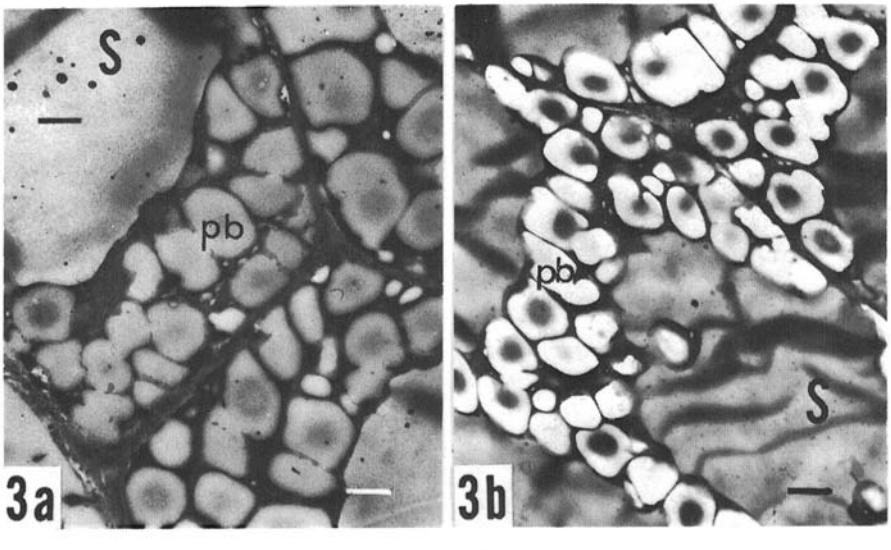

Fig. 3. Endosperm protein of normal corn, W64A inbred variety. Formaldehyde and osmium tetroxide (×6000). (a) Untreated; (b) treated with ethanol.

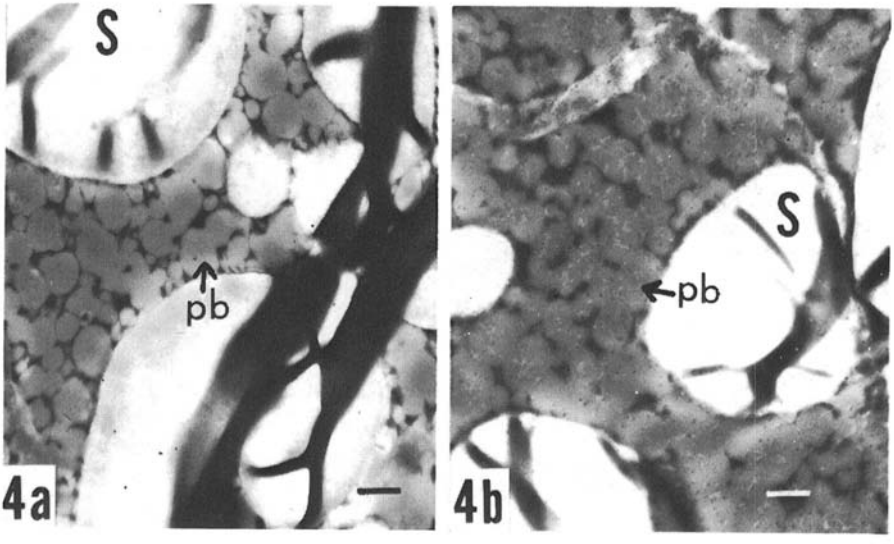

Fig. 4. Endosperm of high-amylose inbred corn, W64A*ae*. Formaldehyde and uranyl acetate (×5700). (a) Untreated; (b) treated with ethanol.

Line scale = 1 μm; S = starch granules; pb = protein body.

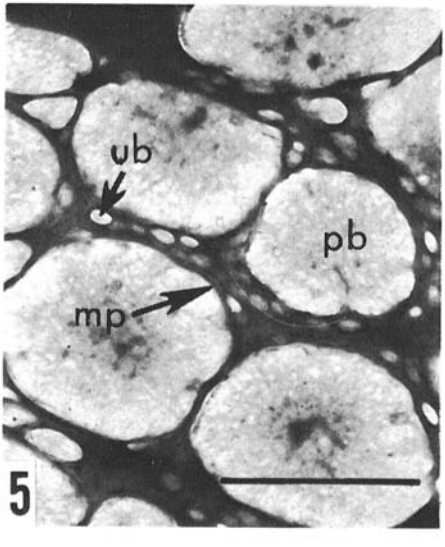

Fig. 5. Endosperm of high-amylose inbred corn, W64A*ae*, showing reticular structure of protein bodies after extraction with ethanol. Formaldehyde and lead citrate (×35,000).

represents at least one additional protein. Electron-dense granules ranging from 0.06 to 0.10 μm in diameter, frequently seen in concentric arrangement in the protein bodies, are a common feature of the insoluble central core (Fig. 2b). Although it is only faintly differentiated in the unextracted bodies, the central core absorbs electron stains heavily after alcohol extraction (compare Figs. 2a and 2b; 3a and 3b).

High-Amylose Corn

After alcohol-extraction, protein bodies of the high-amylose inbred line W64A*ae* show only a small change in stain density (compare Figs. 4a and 4b) and no localization of components within the body on the basis of alcohol solubility. The results of the alcohol treatment also suggest a lower zein content in the bodies than in those of normal corn, but it is impossible to assign a quantitative value on the basis of differences in stain density alone since depth of staining of residual material is greatly intensified by alcohol treatment. Stained with lead citrate, the residual, alcohol-insoluble protein appears as a network (Fig. 5). Apparently the spaces within the network were filled with zein before extraction. Some of the bodies show dense-staining central granules somewhat like those observed in the normal counterpart, W64A.

Subcellular structure of the endosperm in the amylomaize hybrid is shown in Figs. 6a and 6b. As in the high-amylose inbred, W64A*ae*, the alcohol-insoluble residue in the protein body is distributed throughout the body; but unlike W64A*ae*, the residue in bodies of the hybrid is in the form of granules of varying electron density.

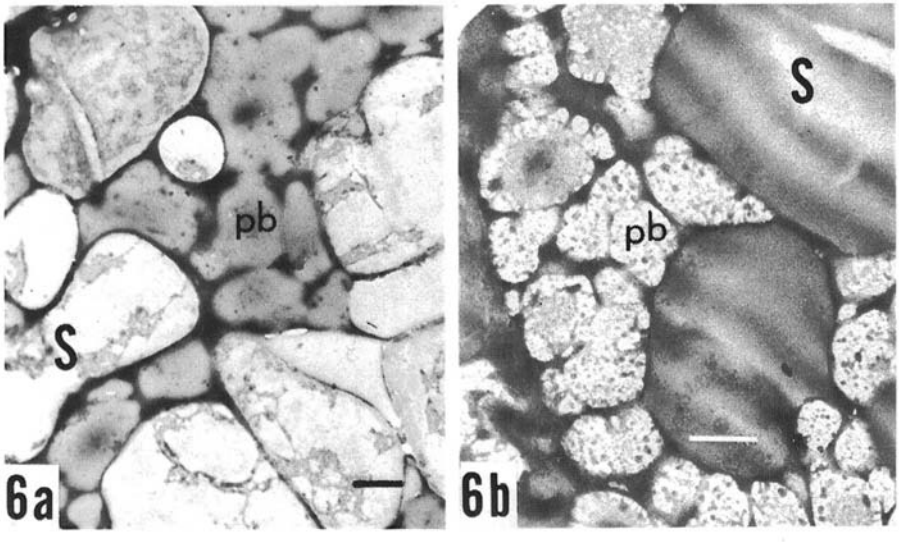

Fig. 6. Endosperm of high-amylose hybrid corn, class 8. Formaldehyde and uranyl acetate. (a) Untreated (×6800); (b) treated with ethanol (×9900).

Line scale = 1 μm; S = starch granules; pb = protein body; mp = matrix protein; ub = unidentified body.

The effect of alcohol extraction on the pattern of protein distribution is observed only as a partial clearing of the protein bodies. A similar change does not occur in other sites. Both ordinary corn and amylomaize contain an electron transparent body about 0.20 to 0.25 μm in diameter (Fig. 5) dispersed in the glutelin protein, the matrix which fills the space around the zein bodies. This body was reported previously in mature ordinary maize (8). It is apparently digestible with Pronase but its solubility in ethanol or other protein solvents is unknown.

DISCUSSION

Degradation of the contents of the protein bodies by Pronase, including the alcohol-insoluble fraction, indicates that the material is largely protein; however, the exact composition of the residual alcohol-insoluble material is uncertain. Presence of small amounts of nucleic acid and phospholipid, in addition to protein, is not excluded since the stains we used react with these substances as well as with protein. The pattern of protein distribution, which we noted within protein bodies of mature maize, arises late in kernel development. At some time between 25 and 50 days after pollination, dense-staining granular material which separates from the optically homogeneous protein content collects in the center of the body (8) to form the alcohol-insoluble core of the mature protein body. A similar study of immature amylomaize has not been made. However, the present work suggests that protein body development in amylomaize may follow the same pattern as in normal corn but at a slower rate. Condensation of granular, alcohol-insoluble protein may be delayed in amylomaize so that at kernel maturity the material is just beginning to accumulate at the center of the protein body. The average protein body diameter of 1.16 μm which we found in amylomaize is intermediate between that of normal maize and *opaque-2* (9). In agreement with the relatively small size of the protein bodies, lysine content of amylomaize hybrids is about 50% above that of ordinary dent hybrid corn (3). However, in the high-amylose inbred variety, W64A*ae*, the lysine level is not significantly higher than that of normal corn (3). This difference in lysine content between the high-amylose hybrid and the inbred variety suggests a corresponding difference in both composition of the protein bodies and in their number.

Protein bodies of corn endosperm are commonly regarded as the site of zein, a storage protein with an unfavorable amino acid balance, while the more nutritionally adequate proteins are found exclusively in the matrix outside of the bodies. However, electron micrographs suggest that the amount of nonzein, ethanol-insoluble protein present in the bodies is significant. Christianson *et al.* (10) found that free protein bodies isolated from immature (24 days after pollination) ordinary maize are distinctly higher in essential amino acids, particularly in methionine, than is the zein fraction alone. These authors did not determine how much of the protein in the immature bodies was alcohol-soluble; however, their data suggest that the favorable amino acid balance of the whole body proteins must have been due to alcohol-insoluble material similar to that which we observed in electron micrographs of mature, ethanol-extracted protein bodies.

Examination of numerous light and electron micrographs of normal and high-

amylose corn suggests that a large proportion of the total endosperm storage protein in both normal corn and amylomaize must be bound within the bodies. Consequently, the composition and availability, particularly of the alcohol-insoluble component of the bodies, assume importance nutritionally. In grits from normal corn, Spanheimer *et al.* (11) showed that Pronase and other proteases failed to solubilize the alcohol-soluble protein. Their work suggests that if the zein was not solubilized by enzymes, other, possibly more nutritious, components of the zein bodies were likewise unavailable nutritionally. Intact protein bodies, such as those likely to occur in grits, are protected by a membrane (8) which apparently interferes with penetration of enzymes. Our present studies with sections much thinner than the average diameter of the zein bodies demonstrate that protein body contents are readily solubilized by Pronase when the membrane covering the body is removed.

Literature Cited

1. ZUBER, M. S., GROGAN, C. O., FERGASON, V. L., DEATHERAGE, W. L., and MacMASTERS, M. M. Some observed secondary effects of high-amylose genes in maize. Cereal Chem. 37: 212 (1960).
2. BAUDET, J., CAUDERON, A., FAUCONNEAU, G., MOSSÉ, J., and PION, R. Sur un troisieme gene mutant (amylose extender) qui accroit la Teneur en lysine du grain de Mais et sur son effect cumulatif avec le gene *opaque-2*. C. R. Acad. Sci. Ser. D 266: 2260 (1968).
3. WOLF, M. J., HARRIS, C. C., and DONALDSON, G. L. Corn endosperm: Protein distribution and amino acid composition in amylomaize *vs.* normal dent hybrid. Cereal Chem. 53: (1976).
4. SPURR, A. S. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31 (1969).
5. VENABLE, J. H., and COGGESHALL, R. Simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25: 407 (1965).
6. WOLF, M. J., KHOO, U., and SECKINGER, H. L. Subcellular structure of endosperm protein in high lysine and normal corn. Science 157(3788): 556 (1967).
7. WOLF, M. J., and KHOO, U. Mature cereal grain endosperm: Rapid glass knife sectioning for examination of proteins. Stain Technol. 45(6): 277 (1970).
8. KHOO, U., and WOLF, M. J. Origin and development of protein granules in maize endosperm. Amer. J. Bot. 57(9): 1042 (1970).
9. WOLF, M. J., KHOO, U., and SECKINGER, H. L. Distribution and subcellular structure of endosperm protein in varieties of ordinary and high-lysine maize. Cereal Chem. 46: 253 (1969).
10. CHRISTIANSON, D. D., NIELSEN, H. C., KHOO, U., WOLF, M. J., and WALL, J. S. Isolation and chemical composition of protein bodies and matrix proteins in corn endosperm. Cereal Chem. 46: 372 (1969).
11. SPANHEIMER, J., FREEMAN, J. E., HEADY, R. E., and HEADLEY, V. E. Air classification of corn grits. I. Softening grits with enzymes and chemicals. Cereal Chem. 49: 131 (1972).

[Received January 3, 1975. Accepted April 9, 1975]