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Subcellular localization of glutelin-2 in maize *(Zea mays L.)* endosperm

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Abstract

Accumulation of the 28 KD protein of the glutelin- (G2) fraction was followed in developing maize endosperm, using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and peak integration of scanned gels. 28 KD glutelin-2 could already be observed from 15 days after pollination and its accumulates reached a plateau during the second half of the development period. The process of biosynthesis of 28 KD glutelin-2 and zeins occurs in a parallel way. Subcellular fractions obtained from linear sucrose gradient centrifugation of developing maize endosperms were analyzed by SDS-PAGE and immunoblotting using a serum reacting against glutelin-2 and 14 KD Z2. Glutelin-2 was found to be present in the protein bodies when subcellular fractionation was carried out without dithiothreitol (DTT). The presence of a reducing agent causes the elution of glutelin-2 from protein bodies. Immunocytochemical labelling using the protein A-colloidal gold technique in protein bodies incubated with anti-G2 IgG revealed that G2 is located mainly in the periphery of protein bodies. These results are interpreted as indicating a structural role for glutelins in protein bodies.

Introduction

Recent studies have yielded a great deal of information on cereal storage proteins. In maize the attention has been primarily focussed on zeins, the major protein fraction (65%) of the endosperm. Zeins are proteins soluble in organic solvents such as 70% ethanol and several polypeptides having molecular weights from 10 to 22 KD have been described from electrophoretical studies (8). During endosperm development, zeins accumulate from around 15 days after pollination and all polypeptides of this fraction are synthesized by membrane-bound polysomes and stored in protein bodies (1) where these proteins are exclusively found (12). The genes coding for some of zein polypeptides have been cloned (9) and it has been shown that they represent a multigene family of proteins scattered along the genome (15, 20).

Glutelins are the second largest group of seed proteins (around 35 to 45%) in maize endosperm. They have not received as much attention as zeins, partly because they constitute a rather heterogeneous group. However a glutelin subfraction called glutelin-2 (G2), prepared by extraction of defatted flour in alkali solution in the presence of a reducing agent, is a better defined group of polypeptides, accounting for around 15% of total endosperm protein. It is composed of a major polypeptide around 28 KD and a second component of 58 KD.

The function of G2 in maize endosperm is not clear neither is its relation with zeins. While several authors found only zeins in protein bodies (2, 13, 21), Vitale *et al.* (22) recently found a 28 KD nonzein protein in this subcellular fraction. The purpose of the present article is to get information about G2 that could contribute to understand their function in maize endosperm. Two kinds of experiments have been carried out, first the study of the rate of synthesis of the glutelin-2 component of

28 KD during maturation of endosperm and second, localization of these proteins in subcellular fractions by use of immunological methods.

Material and methods

Measurement of protein accumulation

Endosperms (ten seeds in each case) from 15, 18, 21, 26, 32 and 44 days after pollination (DAP) and mature double hybrid E-10 maize were used in this study. All series of endosperms were homogenized in a Virtis 45 homogenizer in the presence of acetone/hexane 51:49 at 4 °C. Total proteins were extracted from 25 mg of air dried flour with 0.5 ml sample buffer (0.25 M Tris-HCl pH 6.8, 5% sodium dodecyl sulphate (SDS), 5% 2-mercaptoethanol) for 2 h at room temperature and centrifuged for 10 min at 8 000 **g**. Aliquots of the supernatants were directly loaded on SDS-PAGE (10) gel slabs containing 15% acrylamide and a 150/1 acrylamide/bis-acrylamide relation. Silver stain (14) was used to detect proteins in the gel. The gels were scanned using a Chromoscan 3 Joyce Loebl densitometer. The values of peak integration were multiplied by the endosperm dry weight and plotted against days after pollination. The results are average values of three different experiments.

Subcellular fractionation

Five g of dissected endosperms from 21 DAP E-10 maize grains stored at -70 °C were ground in a mortar in the presence of 5 ml of buffer A (100 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM EDTA, 10 mM MgCl2) containing 10% sucrose and filtered through a nylon cloth and centrifuged at 500 **g**. Homogenization and subsequent treatments were performed at 4 °C. 0.5 ml aliquots were layered on 12 ml linear 20 to 70% sucrose gradients in buffer A and centrifuged for 12 h at 230 000 **g** using a Beckman SW40 rotor. The gradients were monitored at 254 nm using a ISCO UA-5 monitor and 0.5 ml fractions were collected. Fractions were pooled and they were diluted in five volumes of buffer A and centrifuged for 4 h at 100 000 **g**. The pellets were stored at -70 °C to be used in further experiments.

Antisera

Antisera against purified G2 were raised in rabbits as described previously (3). The IgG fraction was obtained by DEAE-cellulose chromatography (Whatman DE-52) and it was lyophilized, dissolved in PBS buffer (10 mM sodium phosphate, 0.15 M ClNa pH 7.3) and stored at -20 °C. Nonimmune sera were employed in control experiments. Antiserum against G2 reacts with G2 proteins: 28 KD and 58 KD, and with a 14 KD maize endosperm protein from the zein fraction as judged by protein blotting. Antiserum against G2 shows no reaction with high molecular weight zein polypeptides.

Immunoblotting

Following the SDS-PAGE, the separated proteins were electrophoretically transferred for 2 h at 60 V and 10 °C to nitrocellulose sheets as described previously (17) by using a BioRad Trans-Blot device. The sheets, preincubated in PBS buffer with 0.05% Nonidet P-40, 0.02% sodium azide, 3% bovine serum albumin, were incubated at 37 °C overnight with anti-G2 serum (dilution 1:50) and extensively washed with 1 M NaCl in PBS. A fluorescein-labelled pig anti-rabbit IgG (Dako-immunoglobulins) was used for the antibody detection.

Electron microscopy and immunocytochemical labelling

Protein body fractions isolated in the presence and in the absence of 1 mM DTT were fixed for 1 h with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, and postfixed with 1% OsO4 in the same buffer. Fixed samples were then washed in cacodylate buffer, dehydrated in a graded series of acetone and embedded in Araldite-Epon. Thin sections were stained with uranyl acetate and lead citrate and viewed with a Philips EM 301 electron microscope.

The preparation of the protein A-gold complex (pAg) and subsequent labelling were carried out following the procedure described by Slot & Geuze (19) with minor modifications. The protein body fraction was fixed by immersion in 0.3% glutaraldehyde in PBS for 5 min at room temperature. The preparation was then neutralized with 0.2 M NH_4Cl in PBS for 10 min, carefully washed with PBS and

dehydrated in a graded series of ethanol at -20 °C. After infiltration in Lowicryl K4M at -20 °C, polymerization was performed by ultraviolet irradiation for 2 days at -20 °C and 1 day at room temperature (18). Thin sections of protein body material mounted on nickel grids were incubated with 1% ovoalbumin and anti-G2 IgG fraction (diluted between 1:30–1:500) at 4 °C overnight. After a quick wash in PBS, the grids were incubated with pAg complex solution in PBS containing 1% ovoalbumin for 1 h at room temperature. The grids were thoroughly washed in PBS, rinsed with distilled water and dried. After staining with uranyl acetate the protein body preparations were viewed in a Jeol electron microscope. In order to demonstrate the specificity of the labelling, two control experiments were performed: i) without serum and ii) with a nonimmune serum.

Results

Rate of accumulation of glutelin-2 in maize endosperm

In order to gain some information on the role of the main polypeptide present in the glutelin-2 fraction, its rate of accumulation in maize endosperm

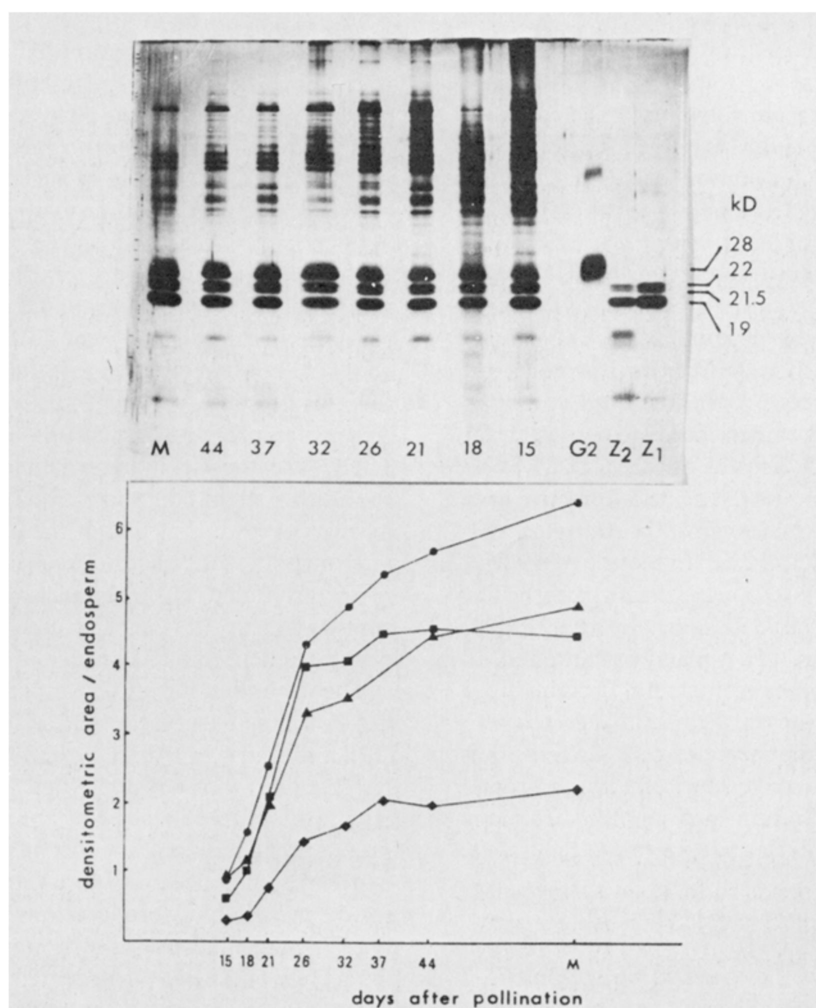

Fig. 1. Accumulation of glutelin-2 compared to zeins during maize endosperm development. Total proteins from 25 mg developing endosperm flours were completely extracted with 0.5 ml of 0.25 M Tris-HCl pH 6.8, 5% SDS, 5% 2-mercaptoethanol. The integral peaks from densitometric scannings were corrected according to dry endosperm weights. ■ 28 KD G2; ● zein-19 KD; ▲ zein-22 KD; ◆ zein-21.5 KD.

has been studied and compared with the results obtained for zeins. A differential extraction procedure has been used by different authors in order to study the rate of synthesis of different protein subfractions in cereal endosperm (8, 4). In our hands the application of this procedure to G2 produces results with little quantitative meaning. It can be observed that in the zein-2 subfraction some protein electrophoretically identical to the 28 KD glutelin is extracted therefore probably corresponding to the same protein. This effect occurs at a different proportion at different days of grain maturation. Furthermore the protein remaining in the final residue seems to vary between different days. These effects are especially important when small scale analysis is used as in our case.

The procedure chosen for our analysis (Fig. 1) includes the extraction of total endosperm protein with SDS, and their separation by gel electrophoresis in conditions where the G2 polypeptide of 28 KD is well resolved. Quantitation of the amount of protein by densitometric analysis of the stained gel bypasses the need for a sequential extraction procedure. It is important to note that in this way the proteins are extracted in the presence of SDS, conditions where endosperm proteins are well soluble and that are normally used to extract proteins not extractable with other procedures. The method is useful when measurements of relative values for different proteins (28 KD G2, and 22 KD, 21.5 KD and 19 KD zeins) expressed as densitometric area per endosperm is plotted against the time after pollination. It is clear that the accumulation of the four proteins studied follows a very similar pattern. The proteins are synthesized between 15 and 37 days after pollination and then a plateau is attained. In the same figure a typical gel of total proteins from endosperm is also shown. From the gel, it is also possible to observe that proteins with lower mobility than those analyzed by densitometry and corresponding to the albumin and globulin fractions decrease in intensity relative to zeins and glutelins.

Subcellular localization of glutelin-2

In order to study the localization of glutelin-2 within endosperm cells, 21 DAP endosperms, were homogenized and fractionated on a linear sucrose gradient. This is a period when the accumulation of these proteins increases most rapidly (see Fig. 1). The sucrose density gradient centrifugation resulted in the profile shown in Fig. 2a which corresponds to the pattern previously described (2). Several fractions were pooled. The first one (T) corresponds to material remained at the top of the gradient composed of no particulate components and proteins solubilized from organelles. The peaks at densities 1.15 (A) and 1.17 g/ml (C) correspond to smooth ER and mitochrondria respectively. Electron micrographs revealed that the highest density peak (1.23 g/ml, fraction F) contained isolated protein bodies (approx 1 μm in diameter) free from contaminating organelles (Fig. 4). Intermediate fractions such as B, D and E were also pooled.

The results of SDS-PAGE of the gradient pooled fractions are shown in Fig. 2b. Components with the same electrophoretical mobilities as glutelin-2 were clearly detected in the fractions from the protein body peak (lane E and F) in addition to the zein polypeptides. Immunoblotting analysis was used to confirm that the G2 proteins accumulate in protein bodies (Fig. 2b). In the same figure, control samples of zeins and G2 are also shown. Incubation with anti-G2 serum followed by treatment with fluorescein-labelled swine anti-rabbit IgG revealed the presence in lanes E and F of both the 28 KD and 58 KD G2 polypeptides (the latter is better seen in the blot presented in Fig. 3) as well as the 14 KD zein which cross-reacts with the anti-serum used. The weaker signals in lanes D and C could correspond to a slight contamination of these fractions by protein bodies although the presence of small amounts of G2 in other organelles cannot be completely excluded. No reaction with the anti-G2 serum was observed when the fractions from the top of the gradient was analyzed.

The accumulation of G2 in protein bodies contrast with the observations of Larkins & Hurkman (13) and Burr & Burr (1) who found only zein polypeptides within protein bodies. Glutelin-2 proteins are soluble in aqueous buffers only in the presence of a reducing agent. The use of DTT in the experimental procedure followed by these authors for the protein body preparations suggests that this reducing agent causes the removal of glutelin-2 from these organelles. In order to confirm this point the homogenization and subcellular fractionation was repeated as described above, but in the presence of 1 mM DTT. Results are shown in Fig. 3. Both SDS-PAGE and subsequent immunoblotting

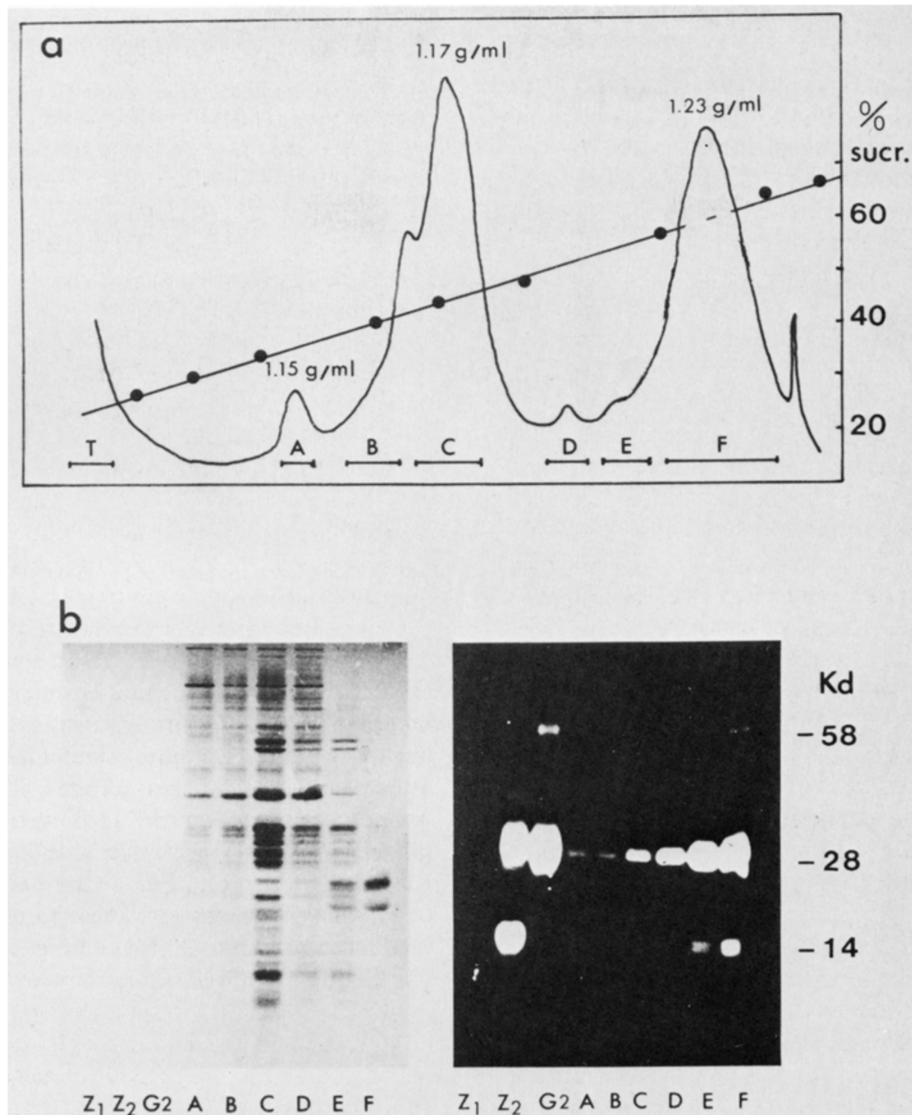

Fig. 2. a) Linear sucrose gradient (20–70%) profile of 21 DAP maize endosperm homogenates in buffer A. After centrifugation 12 h at 230 000 **g** the gradients were monitored at 254 nm and 0.5 ml fractions were collected. The fractions were pooled as depicted in the figure. A–F pooled fractions were diluted in buffer A, pelleted at 100 000 **g** and resuspended in sample buffer.

b) SDS-PAGE and immunoblotting of proteins from sucrose gradient fractionation of a 21 DAP endosperm homogenate. E and F: protein body fraction. Z1, Z2 and G2: zein-1, zein-2 and glutelin-2, respectively, purified from mature endosperms. Z2 were slightly contaminated with G2. No DTT was used in this subcellular fractionation experiment. DAP: days after pollination.

clearly show the almost complete absence of G2 in the protein body fractions (lanes E and F). In contrast, fraction T, which corresponds to the top of gradient, exhibits the two 28 and 58 KD glutelin-2 polypeptide bands. In Fig. 4 the morphological effects of DTT treatment on protein bodies are shown. It can be seen that, in the absence of the reducing agent (Fig. 4a), the protein bodies have a regular shape. When treated with DTT (Fig. 4b) some of them are partially disrupted and others show an altered membrane structure.

Once glutelin-2 polypeptides were detected in the protein body fraction, experiments were carried out in order to locate the position of these proteins within the protein body as accurately as possible and even to discard eventual formation of unspe-

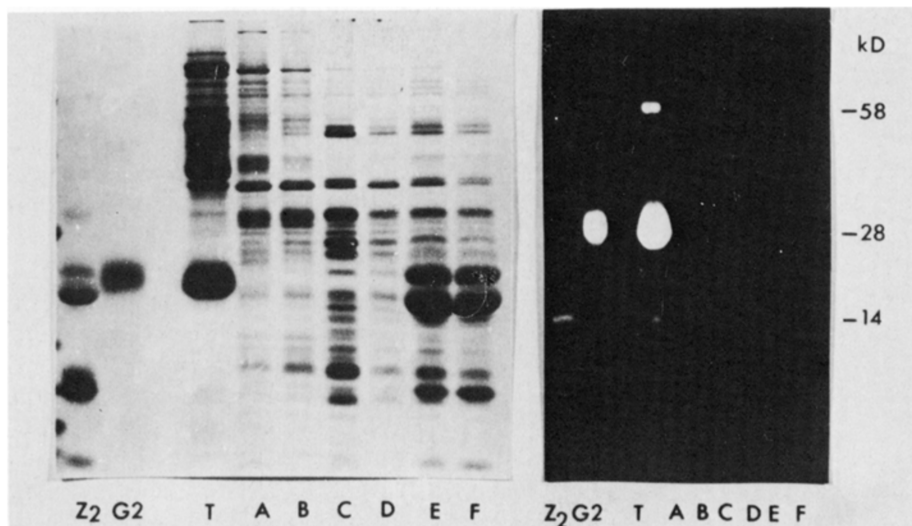

Fig. 3. SDS-PAGE and immunoblotting of proteins from sucrose gradient fractionation in the presence of 1 mM DTT. Lane T: top of the gradient. Z2 and G2: zein-2 and glutelin-2, respectively, purified from mature endosperms.

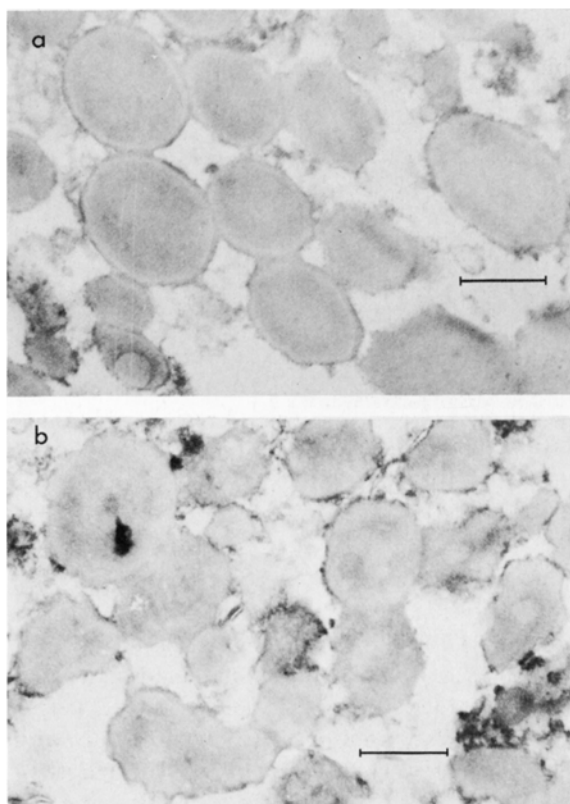

Fig. 4. Electron micrographs of protein bodies isolated in the absence (a) and in the presence (b) of 1 mM DTT. Protein body fractions were fixed in 3% glutaraldehyde, postfixed with 1% OsO4 and embedded in Araldite-Epon. Thin sections were stained with uranyl acetate and lead citrate (Bar: 0.5 μm).

cific aggregates which could co-migrate with protein bodies in the sucrose gradients. Immunocytochemical labelling studies were carried out in an attempt to answer these questions. Protein bodies prepared in the absence of DTT were fixed at low glutaraldehyde concentrations and embedded in Lowicryl 4KM resin in order to retain the protein antigenicity of the sample. These preparations were incubated with anti-G2 IgG and treated with protein A-gold complex. As is shown in Fig. 5, glu-

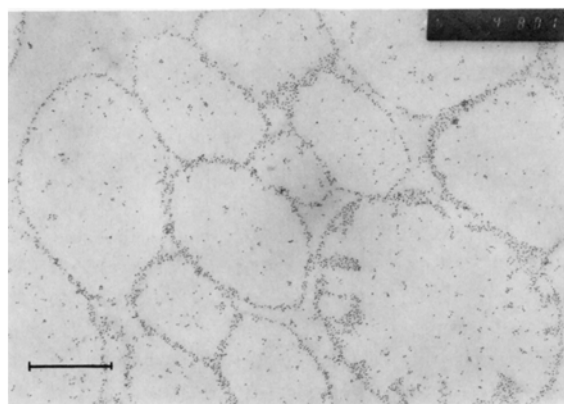

Fig. 5. Immunocytochemical labelling of glutelin-2 in maize endosperm protein bodies. Protein body fraction was fixed in 3% glutaraldehyde, dehydrated in a graded ethanol series and embedded in Lowicryl K4M at -20 °C. Incubation with anti G2-IgG and protein A-gold labelling are described in the text (Bar: 0.5 μm).

telin-2 polypeptides are almost exclusively located in the periphery of the protein bodies. No significant amount of label can be observed in the interstitial gaps. No label was observed in association with protein bodies when control experiments were done with non immune serum IgG or in the absence of IgG (not shown). The results obtained allow to exclude the possibility that the G2 co-sediment in the gradient with the protein body fraction due to an effect of unspecific aggregation.

Discussion

The 28 KD G2 polypeptide is the major non-zein protein in the maize endosperm cell. The same protein probably corresponds to the alcohol-soluble reduced glutelin (ASG) studied by Esen *et al.* (6) or the reduced soluble protein (RSP) studied by Vitale *et al.* (22). A protein having the same mobility is also present in the glutelin-1 fraction described by Landry *et al.* (11). The accumulation of the 28 KD polypeptide as a function of the time after pollination has been studied in the present work by integration of the corresponding electrophoretic band in total endosperm protein analysis. The results for this protein have been compared with those obtained by a similar analysis carried out with zeins. The results show a correlation in the synthesis of the two protein fractions, while proteins having a lower mobility show a clear decrease in intensity during grain maturation in agreement with the results obtained by other authors (4) (5). This observation suggests either a similar function of the two types of proteins as storage elements, or an structural function of glutelin-2 proteins required for the storage of zeins. The other results presented in this paper point in favour of the second hypothesis.

Zeins are synthesized in membrane-bound polysomes and are stored within protein bodies. Our results indicate a similar subcellular localization of glutelin-2 proteins. The fractionation of subcellular components in the absence of DTT and the identification of glutelin-2 polypeptides by immunoblotting allows a clear demonstration that these proteins accumulate in the protein body fraction. In the presence of the reducing agent the proteins are solubilized in agreement with the results of Burr & Burr (2) who, by using DTT, found only zeins in these fractions and also in agreement with those of Vitale *et al.* (22) who found specific solubilization of RSP proteins (equivalent to glutelin-2) in the presence of a reducing agent.

Heterogeneity in the protein components of protein bodies has been reported for cereals other than maize, for instance in *Avena sativa* (16). In maize, we show now that, in addition to zeins, protein bodies contain also glutelin-2 polypeptides. By using the protein A-colloidal gold technique in protein bodies incubated in the presence of anti-G2 IgG these proteins can be localized mainly in the periphery of protein bodies. This is the first report, to our knowledge, of the use of this technique in maize. The small size of the label (15 nm approx.) makes this procedure specially suitable for studies in subcellular fractions such as protein bodies. It has to be pointed out that the antibodies used have a cross-reaction with the 14 KD band of zein-2. It is possible that some of the grains observed in the central part of the protein body sections correspond to reaction with this protein. However, the immunological relation of these two proteins and their common solubilities in the presence of a reducing agent could also indicate a similar function. The sequential extraction procedure is an arbitrary system of classification of endosperm proteins and our results suggest that the 14 KD protein could be better classified in the same group of the 28 KD G2.

After solubilization of the 28 KD protein by DTT, protein bodies undergo a change in their general shape as observed by electron microscopy indicating a loss of internal structure. Esen *et al.* (7) have sequenced the N-terminal region of proline-rich ASG corresponding to glutelin-2. This sequence includes a hexapeptide sequence Pro-Pro-Pro-Val-His-Leu repeated six times in tandem. Such periodicities can be found in animal sequences of proteins with a structural role (7). Glutelin-2 proteins have also a high cysteine content (6.5%). The formation of intermolecular aggregates of these proteins and with other cysteine-rich protein from endosperm, such as low molecular weight zeins-2 having immunological relationship with G2, could result in a network of proteins maintaining the protein body structure. In this sense the accumulation of these proteins should be parallel to that of zeins as they will be required for the maintenance of the protein body structure.

Acknowledgments

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