Uncleaved legumin in developing maize endosperm: identification, accumulation and putative subcellular localization

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Abstract

While identifying proteins present in the cytoskeleton and protein body fractions from maize (Zea mays L.) endosperm, a 51 kDa protein was discovered in a fraction containing small (~200 nm in diameter) protein bodies. Based on partial amino acid sequences of V8 protease fragments, degenerate primers were made and fragments of cDNA encoding these partial sequences were cloned. Using 3’ and 5’ PCR, a full-length cDNA encoding this 51 kDa protein was obtained, which was identified as legumin-1. In other plants, this protein is generally cleaved into 20 and 35 kDa subunits after synthesis. However, SDS-PAGE of both the native and denatured protein indicates that cleavage does not occur in corn endosperm, even though the cleavage site (asparagine) is conserved. The lack of cleavage is presumably because the canonical cleavage sequence downstream from the cleavage site is absent. Levels of transcript and encoded protein were compared in all three varieties and it was shown that both are more abundant in wild-type maize than in opaque-2 or sweet corn. Finally, using TEM, it was shown that the protein appears to occur in morphologically distinct protein bodies, very similar to the protein bodies in legumes.

Key words: Cytoskeleton, gene expression, legumin, peptide mapping, protein body, zein.

Introduction

Legumin is a member of a family of storage proteins (11S globulins) originally found in the Leguminaceae (Saalbach et al., 1991; Shewry et al., 1995; Shewry and Casey, 1999; Shutov et al., 1995; Wright and Boulter, 1974), but later found in a diverse array of higher plants, including monocots (Katsube et al., 1999; Muntz, 1996; Shewry and Casey, 1999; Shutov et al., 1995; Tai et al., 1999). This histidine- and glutamine-rich protein is synthesized as a single polypeptide of 50–55 kDa in which the C-terminus of the α subunit (always asparagine) and the N-terminus of the β subunit (always glycine) are joined by a peptide bond. As the nascent pre-prolegumin is inserted into the ER lumen, the α and β subunit regions are linked via cysteine residues by disulphide bonds, the signal sequence cleaved, and prolegumin generated. The prolegumin is assembled into trimers, transferred through vesicles into the storage vacuole, and cleaved into α and β subunits to form a hexamer (Muntz, 1996). Molecular masses of α and β subunits are generally 30–40 kDa and 18–20 kDa, respectively (Muntz, 1996; Wright and Boulter, 1974; Saalbach et al., 1991; Shewry et al., 1995; Shewry and Casey, 1999). The amino acid sequences of β chains are more homogeneous than those of the α chains, which vary considerably in length because of different numbers of repeats in the C-terminal region (Muntz, 1996; Shewry et al., 1995; Shewry and Casey, 1999).

The transcript encoding a legumin-like protein has been identified in cDNA libraries from both wild-type (B73)
Maize endosperm (Woo et al., 2001) and from sweet corn (DDBJ/EMBL/GenBank Accession number AB073081) and show 96% identity and 97% similarity. These sequences also indicate that the protein lacks the canonical cleavage site for generating α and β subunits. However, this has not been verified experimentally since the protein itself has never been isolated and identified in maize.

In addition to the 11S globulins such as legumin, there is another closely related storage protein, the 7S globulin, vicilin. This protein forms a trimer after the polypeptide is linked by disulphide linkages and transferred into the storage vacuole (Muntz, 1996; Tai et al., 2001). Vicilins are, however, not cleaved into subunits, thus the structure of maize legumin-1, which lacks the canonical cleavage site, could pose crucial insight into the molecular evolution of both 7S and 11S storage globulins in plants.

It is shown here that a legumin does exist in maize, that it is uncleaved, that it appears to be localized to small protein bodies essentially identical to those found in legumes, and that it is more abundant in wild type (W64A), than in sweet corn or opaque-2 maize. Phylogenetic relations between maize legumin and 7S globulin (vicilin) are also discussed.

Materials and methods

Plant materials

Three varieties of maize (Zea mays L.) including sweet corn (SW, cv. Peter), W64A wild type (WT), and opaque-2 (o2) were field-grown at the Faculty of Agriculture, Ehime University, Japan. Developing seeds were collected at 4, 7, 14, 21, and 28 DAP, immediately frozen in liquid nitrogen and stored at −85 °C until processing.

Fractionation of protein bodies

To furnish various protein body fractions from sweet corn, 14 DAP sweet corn endosperm was homogenized in 10 vols of cytoskeleton-stabilizing buffer (CSB) consisting of 5 mM HEPES-KOH (pH 7.4), 10 mM Mg(OAc)2, 2 mM EGTA, and 1 mM phenylmethylsulphonyl fluoride (PMSF) containing 0.5% polyoxyethylene-10-tridecyl ether (PTE) (Abe and Davies, 1995; Abe et al., 1991), filtered through Miracloth (Calbiochem–Novabiochem Corp. La Jolla, CA, USA). After allowing the bulk of the starch grains to sediment at 1 g for 5 min, the filtrate was centrifuged successively at 30 g for 5 min (protein body fraction 1), 250 g for 5 min (protein body fraction 2), 4000 g for 10 min (protein body fraction 3), and 27,000 g for 15 min (protein body fraction 4). All operations were conducted at 4 °C.

Gel electrophoresis and protein digestion by V8 protease

The amount of protein was determined using a Bio-Rad protein kit (Bio-Rad Laboratories, Hercules, CA, USA) with a DU 640 spectrophotometer (Beckman Coulter, Inc. Fullerton, CA, USA), and electrophoresis was performed as described previously (Abe et al., 1992; Abe and Davies, 1995). Briefly, protein body pellets (45 μg protein) were dissolved in LDS buffer, heated at 95 °C for 5 min, separated by SDS-PAGE on a 15% acrylamide gel, and stained with Brilliant Blue R250. The 27,000 g pellet (protein body fraction 4) was resuspended in CDB (200 mM Tris-HCl (pH 8.5), 450 mM KOAc, 25 mM Mg(OAc)2, 2% PTE (Abe and Davies, 1995) and centrifuged at 27,000 g for 15 min to pellet undissolved materials.

The resultant pellet (protein body fraction 4a) was resuspended in LDS buffer and heated at 95 °C for 5 min, separated on an 8% polyacrylamide gel, and stained with Brilliant Blue R250.

As described previously (Shibata et al., 1999), the protein bands were excised and digested with Staphylococcus proteinase V8 (EC 3.4.21.19) by the Cleveland method (Cleveland et al., 1977) electrophoresed on a 15% polyacrylamide gel, blotted on a PVDF membrane, and stained with Brilliant Blue R250. Distinct peptide fragments in the blot were excised and subjected to N-terminal amino acid sequencing using a protein sequencer (Model HP241, Hewlett-Packard).

Isolation of total RNA from corn endosperm

Maize endosperm was homogenized in 5 vols of RNA extraction buffer, consisting of 0.1 M Tris-HCl (pH 8.0), 12.5 mM EDTA, 0.15 M NaCl, 1% SDS, and 1% β-mercaptoethanol and extracted at least three times with an equal volume of phenol/chloroform/isoamylalcohol (25/24/1 by vol.). The aqueous phase was extracted twice with chloroform/isoamylalcohol (24/1 v/v). The crude total RNA was precipitated from the aqueous phase with 2.5 vols of ethanol at −85 °C for 12 h, and collected by centrifugation at 27,000 g for 20 min at room temperature. The crude total RNA pellet was dissolved in a commercial extraction buffer (Quick Prep Total RNA Extraction Kit from Amersham Pharmacia Biotech), and processed further using the kit according to the manufacturer’s manual.

Cloning of the full length cDNA encoding 51 kDa protein

cDNA was synthesized against total RNA from 14 DAP sweet corn endosperm, using a tagged oligo-dT primer. 5′-AAGAATTCT-CGAGCTCCAGAA-3′. RT-PCR was performed using the tag primer and a degenerate primer whose design was based on the partial N-terminal amino acid sequence of a V8 fragment (Shibata et al., 1999). The fragment obtained was subcloned in pMOSBlue vector using pMOSBlue vector blunt ended cloning kit (Amersham Pharmacia Biotech) and sequenced. 5′-RACE was also used to confirm the 5′ sequence.

Northern blotting

Equal amounts (10 μg) of total RNA extracted from corn endosperm at various time periods were separated by agarose gel electrophoresis under denaturing conditions in the presence of formaldehyde, blotted onto a nylon membrane, and stained to show the amount of RNA loaded. The membrane was then probed with [α-32P]-labelled DNA probe of full-length cDNA for the SW 51 kDa protein. After the legumin probe was stripped off, the same membrane was probed with [α-32P]-labelled DNA probe of a cDNA for SW 27 kDa γ-zein, which was 850 bp long and included the complete coding sequence of the zein (672 bp). Equal specific activities of probes (1.0×106 cpm μg−1) were used for probing legumin-1 and 27 kDa γ-zein. Radioactivity on the probe membrane was visualized and quantified by BAS-2000 (Fuji Film, Co. Ltd, Japan).

Electron microscopy of protein bodies from corn endosperm in situ and in vitro

WT, SW, and o2 corn endosperm (14 DAP) was fixed in 2.5% glutaraldehyde with 100 mM phosphate buffer (pH 7.0), post-fixed with 1% OsO4, dehydrated in an alcohol series, embedded in epoxy resin, cut into 100 nm thick sections, and viewed at 300 kV under a transmission electron microscope (Model JEM-3010/HEC, JEOL). To observe protein bodies in various fractions, protein body pellets were embedded in 1% agarose, fixed in 2.5% glutaraldehyde in CSB, dehydrated, embedded in epoxy resin, and sectioned as described above.
Results

Isolation of a 51 kDa protein from sweet corn endosperm

Sweet corn endosperm (14 DAP) was homogenized in CSB, filtered through Miracloth, separated by sequential centrifugation into different-sized protein body fractions, and proteins in these fractions were separated by SDS-PAGE (Fig. 1A). Although all the zeins are present to various degrees in each of the protein body fractions 1–4 (Fig. 1A, lanes 1–4), there are far less zeins and far more larger molecular mass proteins in the protein bodies (fraction 4) sedimenting at the highest g forces (Fig. 1A, lane 4) and virtually no zeins in the 27 000 g supernatant (Fig. 1A, lane 5). In order to identify novel proteins in fraction 4 protein bodies, the associated ribosomes and the cytoskeleton proteins were removed by resuspending the fraction 4 pellet in a cytoskeleton depolymerizing buffer (CDB) and re-centrifuging it at 27 000 g to obtain a ‘washed’ protein body pellet (protein body fraction 4a). This fraction was subject to SDS-PAGE, and protein patterns are shown in Fig. 1B. There were several abundant proteins in the fraction, including those with apparent masses of 70 kDa (partially sequenced, but not yet identified), 57 (identified as a 50 kDa γ-zein homologue by partial amino acid sequencing: see Table 1) and 51 kDa.

In order to identify this 51 kDa protein, the band was excised from the gel, digested with Staphylococcus...
proteinase V8 (Cleveland et al., 1977) or by CNBr (Jahnen-Dechent and Simpson, 1990), and the fragments separated by electrophoresis (Fig. 2). The undigested protein was essentially pure (Fig. 2, lane 1 'a') and it furnished at least six fragments (Fig. 2, lane 2 'b'–'g') ranging in size from about 7 kDa to 20 kDa. These peptide fragments were subjected to N-terminal amino acid sequencing using a protein sequencer (Model HP241, Hewlett-Packard), and these sequences are shown in Table 1 for the V8 fragments V8-1 (19.6 kDa), V8-2 (19.3 kDa) V8-3 (16.8 kDa), V8-4 (14.8 kDa), V8-5 (14.2 kDa), V8-6 (7.3 kDa), and CNBr-1 (8.0 kDa). All of the fragments were highly homologous (77–100% identity) to a protein that has not yet been isolated, but which has been putatively identified as corn legumin-1 based on its nucleotide sequence (AF371279, AB073081). This strongly suggests that the 51 kDa protein isolated here is a legumin-1.

**Isolation and analysis of a cDNA sequence encoding legumin-1**

To obtain the complete structure of this 51 kDa protein, degenerate primers based on the partial amino acid sequence of V8-5 (DDBJ/GenBank, AB073081) were used first. The cDNA was synthesized using a tagged oligo dT RT- primer, and PCR was performed using the gene specific primer and the tag primer (Shibata et al., 1999). To complete the 5′ sequence, RACE-PCR was performed, and the full-length cDNA sequence is shown in Fig. 3. The cDNA is 1671 nt long with a coding sequence of 1449 nt (from no. 21 to no. 1469) and it encodes a polypeptide of 51 kDa composed of 482 amino acid residues (AB060697). All of the partial amino acid sequences of V8 and CNBr fragments (Table 1), along with their cleavage sites (E for V8 and M for CNBr, in italics), were located in the encoded amino acid sequence (Fig. 3). The encoded amino acid sequence (DDBJ/GenBank Accession number AB073081) was very similar to legumin-1 from wild type (W64A) corn (AF371279), and so the protein can be identified as legumin-1 from sweet corn. It is interesting to note that, according to the nucleotide sequence, the cleavage site for V8-5 was composed of two glutamic acid residues. It is presumed that this means that one of the glutamic acid residues must have been converted into glutamine by post-translational amidation, since V8 proteinase does not cleave EE (Cleveland et al., 1977).

![Image](https://example.com/image.png)

**Fig. 2.** V8-digested fragments of the 51 kDa protein from sweet corn. The 51 kDa protein band shown in lane 1 in Fig. 1B (indicated by b) was excised and digested with *Staphylococcus* proteinase V8 (EC 3.4.21.19) by the Cleveland method (Cleveland et al., 1977) electrophoresed on a 15% polyacrylamide gel, blotted on a PVDF membrane, and stained with Brilliant Blue R250. Lanes are: 1, undigested 51 kDa protein (indicated by 'a' on the left); 2, peptide fragments obtained by V8 proteinase digestion (indicated by: b, 19.6 kDa; c, 19.3 kDa; d, 16.8 kDa; e, 14.8 kDa; f, 14.2 kDa; g, 7.3 kDa); 3, high molecular mass markers (masses shown by long arrows to the right); 4, low molecular mass markers (masses shown in italics and short arrows on the right).

**Table 1. Identification of proteins by peptide mapping and sequencing**

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<tr>
<th>Protein (kDa)</th>
<th>Fragments</th>
<th>Digestion Size (kDa)</th>
<th>Sequence</th>
<th>Source</th>
<th>Identity/similarity (%)</th>
<th>Accession number</th>
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<td>19.6</td>
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<td>77/88</td>
<td>AF371279</td>
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<tr>
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<td>19.3</td>
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<td>legumin-1</td>
<td>80/90</td>
<td>AF371279</td>
<td></td>
</tr>
<tr>
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<td>16.8</td>
<td>VRHHVVRDLQ</td>
<td>legumin-1</td>
<td>80/80</td>
<td>AF371279</td>
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<tr>
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<td>TOQQQYGGYGY</td>
<td>legumin-1</td>
<td>100/100</td>
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<tr>
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<tr>
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<tr>
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Changes in expression of legumin during development

RNA was extracted from WT, SW and o2 corn at 7, 14, and 28 DAP, electrophoresed, blotted and stained to visualize rRNA to verify equal loading (Fig. 5A), probed with the full-length cDNA probe for legumin-1 (Fig. 5B), then with a probe for SW 27 kDa γ-zein (AB086264), which was 850 bp long and included the complete coding sequence of the zein (672 bp) (Fig. 5C), and the amount of each transcript furnished (Fig. 5D). The specific activity of probes used for legumin and γ-zein mRNAs was 1.0 x 10^6 cpm μg^-1. In all varieties, the legumin transcript was low at 7 DAP, increased substantially by 14 DAP and then declined by 28 DAP, while it was highest in WT, lower in o2 and lowest in SW (Fig. 5B). The γ-zein transcript was also highest in WT and continued to increase during development, while it was lower (but continually increasing) in SW, whereas in o2 it had declined substantially by 28 DAP (Fig. 5C). The levels of zein transcript were always much higher than legumin transcript, ranging from 7-fold more (o2, 7 DAP) to 80-fold more (SW, WT, 28 DAP). In general the ratio was much lower for o2, with the highest value being 16 (at 28 DAP), partly because legumin was high in o2, but also because γ-zein was rather low. The legumin probe interacted with just 1 band (of the appropriate size), while the γ-zein probe hybridized with several bands, especially one of about 3500 nt in WT and about 2500 nt in SW.

An attempt was made to quantify the levels of legumin, but since an antibody was not available, this proved rather
difficult. Nevertheless, an estimate of the amount of this protein was made using the ‘washed protein body 4 fraction’, i.e. fraction 4a (Fig. 1B), since it contained most of the legumin protein, and the results are shown in Fig. 6. The 51 kDa band (putative legumin) indicated by ‘a’ was high in this fraction from all three varieties, while the amount of γ-zein (labelled ‘b’) and a partially sequenced, yet still unidentified 70 kDa protein (‘c’) were more variable in amount. It was decided not to quantify these data, since it was not certain that the 51 kDa band was pure, as it has the same electrophoretic mobility as β-tubulin and eEF1α. However, since the cytoskeleton proteins were removed by washing the protein body fraction, and only trace amounts of these proteins were visualized by antibody staining (data not shown), and since the sequenced fragments from this band (Fig. 1B, lane 1; Fig. 2, lane 1) all corresponded to legumin (Table 1), it is presumed that the vast bulk of this protein band is, indeed, legumin.

Ultrastructure of protein bodies in both in vivo and in vitro

With tissue examined in situ, legumin-type protein bodies were found in vacuoles in all varieties, and they were generally less than 200 nm in diameter (Fig. 7A–C). Because of their structural similarity to legumin-type protein bodies seen in other species (Katsube et al., 1999; Tai et al., 2001), it is presumed that these are the ones that contain legumin. Different protein body fractions were examined in vitro, and the ‘normal’ protein body fraction (the 4000 g pellet) contained typical zein storage protein bodies and was associated with ribosomes and, occasionally, with thick filaments and nanotubules (Fig. 7D) as described earlier (Davies et al., 2001). In the (unwashed) protein body fraction 4 (Fig. 7E), there were smaller and fewer protein bodies, again with many ribosomes, isolated small filaments and nanotubules. However, the CDB-treated protein body fraction 4, i.e. the ‘washed’ protein body fraction 4a (Fig. 7F), contained similar protein bodies to those in fraction 4 (Fig. 7E), but also quite numerous legumin-type protein bodies (Fig. 7F) similar to those shown previously for storing legumin in rice and other plant species (Katsube et al., 1999; Tai et al., 2001). The average size of protein bodies in protein body fraction 3 was more than 500 nm (Fig. 7D) while those in protein body fractions 4 and 4a were less than 500 nm (Fig. 7E, F). The average size of protein bodies in planta in SW was about 760–860 nm in this study (Fig. 7A), while those in WT and o2 were about 240–320 nm (Fig. 7B, C), which is consistent with those found by previous workers for WT and o2 (Lending, 1996).

Discussion

Background

Legumins constitute a family of seed storage proteins, originally discovered in legumes (Muntz, 1996; Shewry et al., 1995; Shewry and Casey, 1999; Wright and Boulter, 1974) in which the legumin gene is transcribed as a single mRNA encoding a 51 kDa polypeptide. During translation, a disulphide bond is formed, the signal peptide is removed, and the polypeptides form trimers. After synthesis, each
polypeptide is cleaved into α and β subunits at the N-G site of the canonical cleavage motif towards the C-terminus in the storage vacuole (Muntz, 1996). Although legumins have been known for a long time in dicotyledons, especially legumes (Muntz, 1996; Shewry et al., 1995), it was shown only recently that they are also present in monocotyledons such as rice where they accumulate in legumin-type protein bodies, rather than in prolamin-type protein bodies (Okita, 1996). Much more recently, Woo et al. (2001) provided the first evidence for the existence of legumin in maize when they reported that a cDNA encoding a polypeptide with high similarity to legumin was present in the B73 cDNA library. They also showed that this encoded polypeptide contained the asparagine residue where cleavage normally occurs (Woo et al., 2001), but lacked the canonical cleavage motif (Tai et al., 2001) and so they suggested that legumin in maize was not...
cleaved. However, there is no experimental evidence in maize endosperm for the expression of the legumin gene in terms of accumulation of the transcript, its translation into legumin, accumulation of the protein, its molecular mass, or deposition in protein bodies. Since a partial cDNA (AB060697) and the deduced polypeptide (BAB70680) for this putative legumin from maize had already been sequenced, it was decided to find out to what extent the transcript and protein accumulated, whether the polypeptide was cleaved, and where the protein accumulated.

Fig. 7. Ultrastructure of different protein body fractions from corn endosperm in situ and in vitro. (A–C) WT, SW, and o2 corn endosperm (14 DAP) was fixed in 2.5% glutaraldehyde with 100 mM phosphate buffer (pH 7.0), post-fixed with 1% OsO₄, dehydrated in an alcohol series, embedded in epoxy resin, cut into 100 nm thick sections, and viewed under a transmission electron microscope at 300 kV. The storage zone shows legumin type protein bodies (arrows) from SW (A), WT (B), and o2 (C). The average size of prolamin type protein bodies (n=50) in SW was about 800 nm, but about 250–320 nm for WT and o2. (D–F) Protein body pellets were embedded in 1% agarose, fixed, dehydrated, embedded in epoxy resin, and sectioned as described above. Protein body fractions are: D, fraction 3 (4000 g pellet); E, fraction 4 (original 27 000 g pellet); F, fraction 4a (the ‘washed’ protein body fraction 4, see Fig. 1 legend). Legumin type protein bodies are indicated by arrows. Bars correspond to 500 nm.
Prolegumin (11S globulin) trimers cannot assemble into hexamers unless the Asn–Gly linkage is cleaved. In contrast to 11S globulins, 7S globulins such as vicilin form trimers without being cleaved (Muntz, 1996; Tai et al., 2001). Accordingly, it is presumed that the structure of maize legumin-1, which lacks the canonical cleavage site and so is not cleaved, will provide important insights into the molecular evolution of both 7S and 11S storage globulins in plants.

Isolation of the putative legumin protein

In this study an approach was chosen that did not rely on generating an antibody (to the native or bacterially expressed legumin protein). Instead it was speculated that the protein was most probably not cleaved in vivo and rejoined by disulphide bonds and thus would resist dissociation under denaturing conditions. It was also speculated that it would accumulate in the much smaller, legumin-type protein bodies, rather than the larger prolamin-type protein bodies, as is the case in rice (Okita, 1996). These speculations proved valid. When prolamin-type protein bodies were separated under low speed, the bulk of the zeins were in these fractions (Fig. 1A, lanes 1–3), while less zein was present in the smaller protein bodies sedimenting at higher speed (27 000 g), and a protein of 51 kDa was much more abundant in this fraction (Fig. 1A, lane 4). Since the band at 51 kDa could also contain eEF1α and β-tubulin, both of which have almost identical gel mobility as the putative 51 kDa legumin, and because the prolamin protein bodies are more likely to be associated with the cytoskeleton than are the legumin-type (Okita, 1996), it was decided to remove the ‘contaminating’ cytoskeleton proteins (including eEF1α and β-tubulin) by washing the fraction 4 pellet in the specially-designed cytoskeleton-depolymerizing buffer (Abe and Davies, 1995). This fraction was, indeed, enriched with a band at 51 kDa as well as bands at 57 kDa (zein) and at 70 kDa (Fig. 1B, lane 1). 15% acrylamide gels were used in Fig. 1A, which allowed the retention of all of the zeins, but 8% gels were used in Fig. 1B, which allowed most of zeins to be electrophoresed from the gel (Fig. 1B). When several gels similar to those in Fig. 1B were run, the appropriate bands excised, and the protein again subject to electrophoresis, only one major band was apparent (Fig. 2, lane 1). When the protein in this band was cleaved by V8 proteinase, several quite distinct bands were evident (Fig. 2, lane 2), all of which had highly similar, if not identical, sequences (Table 1) to those expected from the encoding nucleotide sequence. Since all the eEF1α and β-tubulin had essentially been removed by washing the pellet in CDB, and because the only fragments yielded after cleavage corresponded to legumin sequences, it is presumed that this band is predominantly (if not totally) legumin.

Characterization and expression of the legumin transcript

Using the appropriate primers based on the amino acid sequences of the V8 protease fragments (Table 1), the cDNA encoding this 51 kDa protein was constructed (Fig. 3) and it was shown that it is almost identical to the one previously described from B73 endosperm (Woo et al., 2001) and quite similar to legumins from a diverse array of plants (Fig. 4). The cDNA does, indeed, encode a polypeptide lacking the cleavage motif, thus explaining why it resists cleavage in vitro under denaturing conditions (Figs 1, 2, 6), but still retains the disulphide linkage sites, which may, or may not, be formed in vivo. RNA was extracted and it was shown that all three varieties accumulate the legumin transcript (Fig. 5). Although γ-zein transcript tends to keep accumulating during development (Giroux et al., 1994), the legumin transcript is lower early in development (7 DAP), maximal at 14 DAP, but then declines somewhat by 28 DAP (Fig. 5), implying that the greatest accumulation of the protein is likely to occur around or after 14 DAP. There were also larger molecular weight bands of about 1300 nt in sweet corn, and 1300 nt and 2000 nt in the wild type (Fig. 5), one of which might encode the 57 kDa γ-zein homologue found in the small protein body fractions (Fig. 1). Not only does the legumin transcript accumulate in all three maize varieties (Fig. 5), but so does the polypeptide (Fig. 6). It was not possible to quantify this protein, since there was no antibody, and the legumin band might be contaminated by other proteins of the same electrophoretic mobility, such as the cytoskeleton-associated proteins, eEF1α and β-tubulin. However, in order to obtain some quantitative information it was decided to minimize contamination by these cytoskeleton proteins by isolating that fraction most abundant in legumin (fraction 4, Fig. 1A) and removing the contaminating proteins by washing in cytoskeleton-depolymerizing buffer (CDB). When this was done, the ‘washed’ protein bodies yielded high amounts of a band at 51 kDa (Fig. 1B), which was, as expected, almost totally devoid of contamination with eEF1α and β-tubulin (data not shown) and furnished fragments of legumin alone when cleaved by V8 protease or CNBr (Fig. 2; Table 1). Accordingly, it was assumed that the band at 51 kDa present more-or-less equally in all three varieties (Fig. 6) consists primarily of legumin and indicates that the protein does indeed accumulate in vivo.

Subcellular localization of legumin

This putative identification of the 51 kDa band (Figs 1B, 2, 6) as legumin is strongly reinforced by the finding that legumin-type protein bodies can be visualized both in situ and in vitro (Fig. 7). These protein bodies are essentially identical to legumin-type protein bodies, since they are irregular in shape and stain deeply with uranyl acetate and
lead citrate after fixation in glutaraldehyde and osmium tetroxide (Fig. 7), as is the case for legumin protein bodies in other plant species (Katsube et al., 1999; Tai et al., 2001). They are clearly distinguishable from the prolamin protein bodies, which are larger, more spherical and less electron dense (Fig. 7). Even though these small protein bodies are morphologically virtually identical to legumin-type protein bodies, this does not provide indisputable evidence for their identity. For this, immuno-electron microscope observation using antibody to legumin-1 is essential. However, since it has not yet been possible to generate such an antibody, no such definitive evidence can be provided in the present study.

In summary, substantial evidence is furnished for the presence of uncleaved legumin in maize, including (a) direct evidence of a 51 kDa protein that yields fragments of solely legumin polypeptide sequences after treatment with V8 protease (Figs 1, 2; Table 1); (b) indirect evidence from peptide mapping and sequencing (Figs 3, 4); and (c) the existence of legumin-type protein bodies (Fig. 7), which could be partially separated by differential centrifugation from the far more abundant zein (prolamin) protein bodies (Figs 1, 7). Future work will focus on (a) generating antibodies to maize legumin definitively to establish its subcellular location and (b) on determining if the maize legumin forms a trimer or hexamer in the storage vacuole, which will be crucial for understanding the molecular and structural evolution of legumins and vicilins.

References


Abe S, You W, Davies E. 1991. Protein bodies in corn endosperm are enclosed by and enmeshed in F-actin. Protoplasma 165, 139–149.


