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Deposition of Matrix and Crystalloid Storage Proteins during Protein Body Development in the Endosperm of *Ricinus communis* L. cv. Hale Seeds[1]

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ABSTRACT

Protein bodies within the endosperm of castor bean (*Ricinus communis* L. cv. Hale) seeds arise from numerous small vacuoles which progressively become filled with storage protein, of which the crystalloid proteins make up approximately 70%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) shows that the crystalloids are a family of at least four proteins which reduce to two complementary groups after 2-mercaptoethanol treatment. The matrix, which comprises the remainder, has two major components, the soluble albumins and the lectins. The lectins are the only glycoproteins within the mature protein body. Both cytochemical staining and SDS-PAGE indicate that the synthesis of the crystalloid and the majority of matrix proteins begins some 20 days after pollination. Additionally, the crystalloid proteins are synthesized concurrently, whereas there is temporal variation in the synthesis of matrix proteins.

In many seeds, storage proteins are localized within an amorphous matrix contained in spherical organelles called protein bodies (11). Commonly, these proteins are globulins (7). In some seeds, however, inclusions of crystalloid proteins are found embedded in the matrix also. Castor bean belongs to this latter group. The matrix of the protein bodies of castor bean seeds is comprised mainly of two groups of soluble proteins, the 7S lectins and the 2S albumins; globulins are absent (13, 18, 21). A storage role for the albumins has been suggested, since these proteins constitute at least 20% of the extracted mature seed protein (18, 23), and they are mobilized during early seedling establishment (22). The 11S crystalloid proteins, which have globulin-like solubility characteristics, complete the spectrum of storage proteins. Differences in solubility between the matrix and the crystalloid proteins effectively compartmentalize them within the protein body. This compartmentalization of proteins, which is evident in the mature seed, raises questions as to the nature and relative timing of their deposition during seed development. In this study, we show that storage protein deposition occurs after vacuolar subdivision is completed. Additionally, the crystalloid is seen as a family of proteins which are synthesized at the same time, whereas the synthesis of the several matrix proteins is more variable.

MATERIALS AND METHODS

Plant Materials. Mature dry seeds of castor bean (*Ricinus communis* L. cv. Hale) were germinated and grown in pots containing 1:1 peat:sand mixture which was irrigated daily with one-half strength Hoagland solution. Plants were maintained in a controlled environment chamber (Conviron, Winnipeg, Canada, under 23°C days, 18°C nights, and an 18-h photoperiod. Flowering occurred after 60 d. The day of full bloom was recorded for individual flowers and was used as the zero date. To ensure fertilization, stigmas were dusted with pollen at this time. Seeds were collected at 5-d intervals to maturity (60 DAP[3]), and seed staging was carried out in accordance with Greenwood and Bewley (9). For convenience, whole endosperms were removed and dried over silica gel for 7 d prior to protein extraction. The protein profiles obtained using material treated this way were identical to those obtained using either endosperm from freshly harvested seed or endosperm which had been frozen in liquid N_2 immediately after harvest.

Light Microscopy. Median longitudinal slices (1-mm thick) of tissue from the central portion of the endosperm were fixed for 2 h at 23°C, using 3% (v/v) glutaraldehyde in 0.05 M K-phosphate (pH 7.0), dehydrated using a 50 to 100% (v/v) ethanol series, and embedded in 2-hydroxyethyl methacrylate (8). Sections (1to 2-μm thick) were made and micrographed. To aid photography, the background was enhanced by staining mounted sections first with 0.05% (w/v) aqueous toluidine blue. This was followed by protein-staining, using 1% (w/v) $HgCl_2$, 0.05% (w/v) bromophenol blue in 2% (v/v) aqueous acetic acid (16).

Protein Extraction. All procedures were carried out at 4°C. The method of Becker *et al.* (2) was used with modifications. Dried endosperms were ground in a mortar in 0.05 M Na-phosphate (pH 7.5) in a ratio of 1 g dry weight of material to 10 ml extraction buffer. The homogenate was centrifuged at 40,000*g* for 30 min. The supernatant, containing matrix and other soluble proteins, was removed, and the pellet was reextracted three times. The supernatants were combined. The pellet, containing the crystalloid proteins was resuspended in extraction buffer, and the crystalloids were solubilized by boiling for 5 min in an equal volume of 65 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS and 10% (w/v) glycerol. When required, 2.5% (v/v) ME was included in this treatment to reduce disulfide bonds. After treatment, the slurry was centrifuged at 40,000*g* for 30 min, and the supernatant containing the crystalloid proteins was removed. Complete extraction of matrix and crystalloid proteins was achieved, since pelleted residues did not react with the mercuric-bromophenol blue stain

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[3] Abbreviations: DAP, days after pollination; ME, 2-mercaptoethanol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; PAS, periodic acid Schiff.

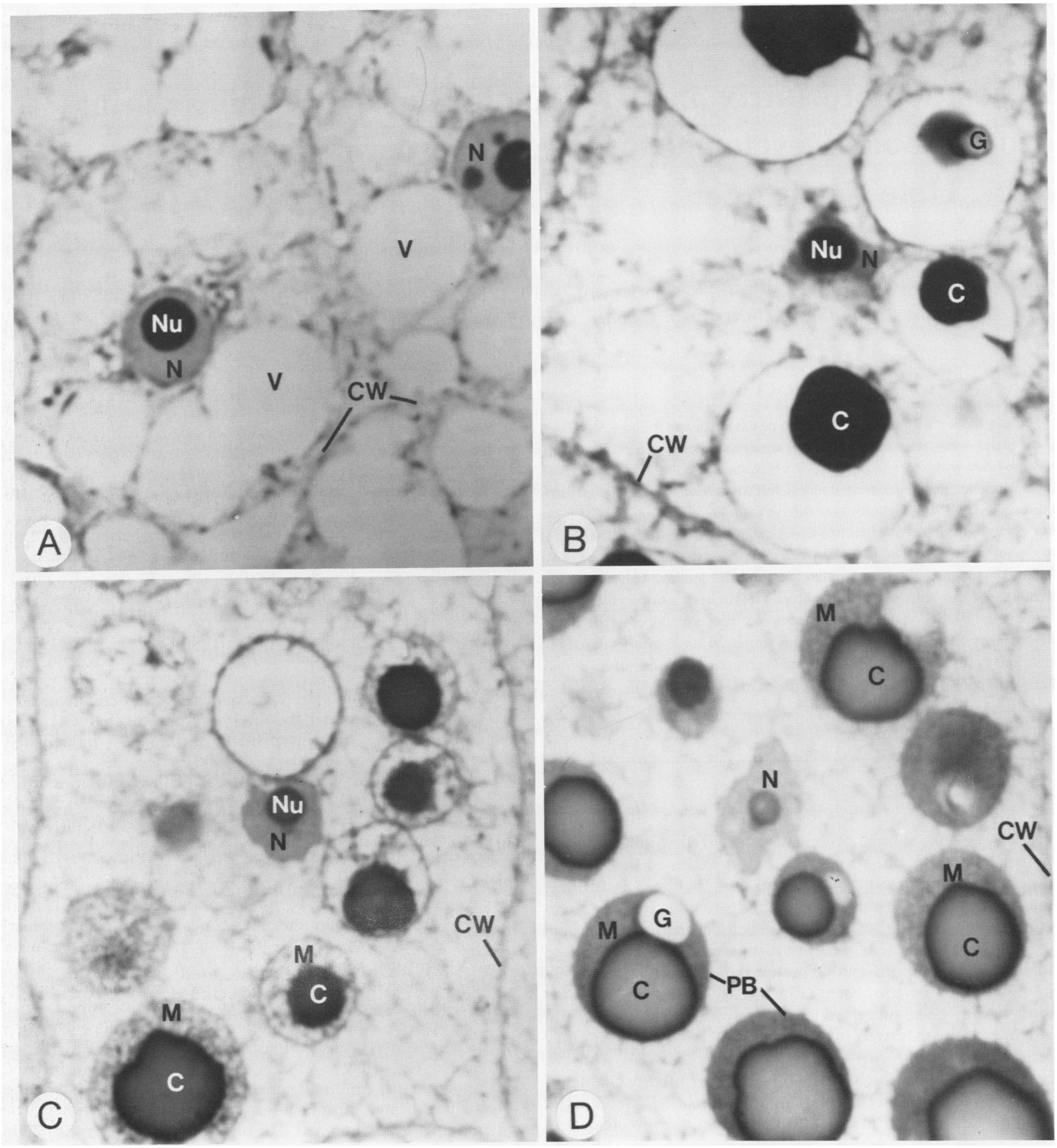

FIG. 1. Light micrographs showing protein deposition during protein body development in central endosperm cells of the castor bean seed. Protein staining procedures are outlined in "Materials and Methods." A, 20 DAP, vacuolar (V) subdivision occurring. Also visible are the nucleus (N), nucleolus (Nu), and cell wall (CW). Note the lack of proteinaceous material within the vacuole. B, 25 DAP, crystalloid protein (C) visible in the subvacuoles. Refractive globoids (G) can also be seen. C, 30 DAP, crystalloids are well developed; an amorphous proteinaceous matrix (M) is also visible. D, 35 DAP, matrix protein deposition virtually complete. At this stage, protein bodies (PB) have the crystalloid, matrix, and globoid components characteristic of protein bodies from mature (60 DAP) castor bean endosprm.

for protein (16). Protein was determined by the method of Lowry *et al.* (12).

Isolation and Fractionation of Protein Bodies. The nonaqueous method of Yatsu and Jacks (20) was used to isolate protein bodies from mature (60 DAP) castor seed endosperm. Subsequent fractionation followed the procedure of Tully and Beevers (18). Since, however, we found that the crystalloid proteins were slightly soluble in glycerol, it became necessary to remove all traces of glycerol prior to extraction in 5M Tris-HCl (pH 8.5). This was achieved by first centrifuging the glycerol-protein body suspension

and then repeatedly washing the pellet with 100% ethanol. After extraction and removal of the soluble matrix proteins, the sedimented crystalloid proteins were resuspended in buffer, and the crystalloid proteins were solubilized as described previously.

SDS-PAGE. Prior to electrophoresis, extracts of matrix and other soluble proteins were boiled for 5 min in an equal volume of 65 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (w/v) glycerol, and 2.5% (v/v) ME (when required). Crystalloid protein solutions were used directly. Electrophoresis was carried out in 12% and 15% gels using a Bio-Rad 220 apparatus (10). Gels were stained for protein with Coomassie blue, after Weber and Osborn (19), and for glycoproteins, according to Zacharius *et al.* (24), omitting periodate from control gels. After drying, the gels were photographed, and printing was controlled to allow resolution of the darkest bands. For mol wt determinations, the gels were calibrated using phosphorylase B, 94 kd; BSA, 68 kd; ovalbumin, 43 kd; carbonic anhydrase, 30 kd; soybean trypsin inhibitor, 21 kd; and lysozyme, 14 kd (supplied by Bio-Rad).

RESULTS AND DISCUSSION

Light Microscopy of Protein Deposition During Protein Body Development. Studies of seeds of *Sinapis* (17) and of several legumes (1, 4–6, 15) have shown that protein bodies within the storage organs arise from numerous small subvacuoles which progressively become filled with storage material. We have found castor bean to be similar in this regard. This is illustrated in the sequence of light micrographs presented in Figure 1 (A–D), which show the appearance of developing central endosperm cells and their protein bodies between 20 and 35 DAP. Although subvacuoles are readily visible within central endosperm cells by 20 DAP (Fig. 1A), proteinaceous material is not detectable within them until 25 DAP. The protein inclusions, seen at 25 DAP (Fig. 1B), have a shape and staining intensity which is characteristic of the crystalloid (18, 21). The matrix proteins in the subvacuoles are not detectable until some 5 d later (Fig. 1C). They stain more lightly with mercuric-bromophenol blue and have a floccular appearance. Protein body formation in central endosperm cells is essentially complete by 35 DAP (Fig. 1D), when a dense crystalloid structure can be seen with a more diffuse matrix. No significant differences were observed between the cell protein bodies at this stage of development and those in the mature seed endosperm (60 DAP).

During this investigation, we observed that the timing of protein deposition was different in different regions of the endosperm. Compared to central endosperm cells, protein deposition occurred some 5 d earlier in endosperm cells at the radicle end and some 5 d later in those at the cotyledon tip. This observation has an important bearing upon the results of the SDS-PAGE experiments (discussed below) which used whole endosperms. Hence, the initial appearance of proteins within extracts of whole endosperms might be expected to reflect most closely their synthesis within the most rapidly developing cells, *i.e.* those at the radicle end of the endosperm. Even so, in all regions, the crystalloid proteins always were detected by staining some 5 d earlier than those of the protein body matrix.

Quantitative Protein Changes during Endosperm Development. Whole endosperms were removed from seeds at different stages of development and dried over silica gel, and crystalloid and soluble proteins were extracted. Because the soluble protein fraction contains proteins other than the matrix storage proteins, this fraction was designed as 'matrix and noncrystalloid.' Quantitative changes in the total amount of this and crystalloid protein fraction during seed development are shown in Figure 2.

After an initial increase during the first 20 DAP (Fig. 2B), the total matrix and noncrystalloid protein declined rapidly on a per g dry weight basis until a constant level was reached at 40 DAP. This decline was because of an increase in dry weight of other

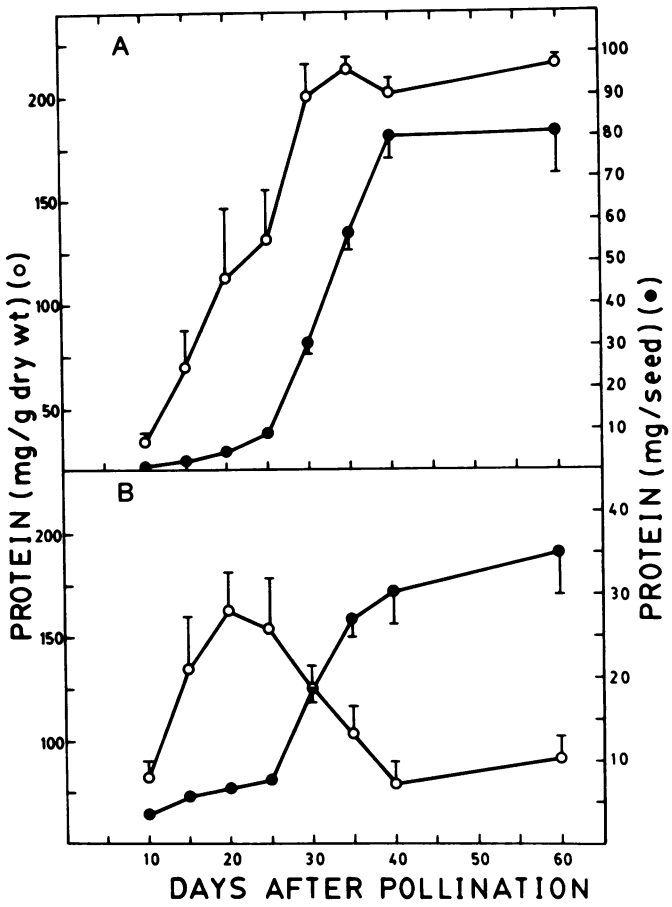

FIG. 2. Quantitative changes in crystalloid protein (A) and matrix and noncrystalloid protein (B) (see text) in extracts of whole endosperms from developing castor bean seeds. Each plotted value is the mean of three determinations ± SD.

storage components after 25 DAP, especially lipids (J. S. Greenwood, unpublished data). On a per seed basis, this protein fraction increased until about 35 DAP, after which little further increase occurred. Similarly, the crystalloid protein fraction (Fig. 2A) increased linearly on a per seed basis over the same time period before becoming constant. This level was maintained to maturity (60 DAP), when the crystalloid proteins comprised some 70% of the total endosperm protein. A similar figure of 73% of total endosperm protein was obtained for the crystalloid protein fraction in protein bodies isolated from the same material. While this is in close agreement with a previous report (18) which used a similar extraction procedure, it is significantly higher than the 42% reported from studies using 1 M NaCl in the extraction buffer (22, 23). However, we found that a substantial amount of proteinaceous material remained within the residue after NaCl extraction of the resuspended crystalloid pellet. This was detectable, using a mercuric-bromophenol blue stain. In contrast, positive staining was not observed if SDS, which has been shown to completely solubilize the crystalloid fraction (18), was included in the extraction buffer.

Qualitative Protein Changes during Endosperm Development.

Protein Body Fractionation. Before examining the deposition of specific matrix and crystalloid storage proteins, we first established their profiles on polyacrylamide gels. Protein bodies isolated from mature seed endosperm (60 DAP) were used, thereby minimizing nonstorage protein contamination. Typical electrophoretic profiles are shown in Figure 3 and their corresponding mol wt in Table I. In the absence of ME, the matrix proteins comprise four groups which are represented by bands 1 and 2, bands 3 to 5, band 6, and

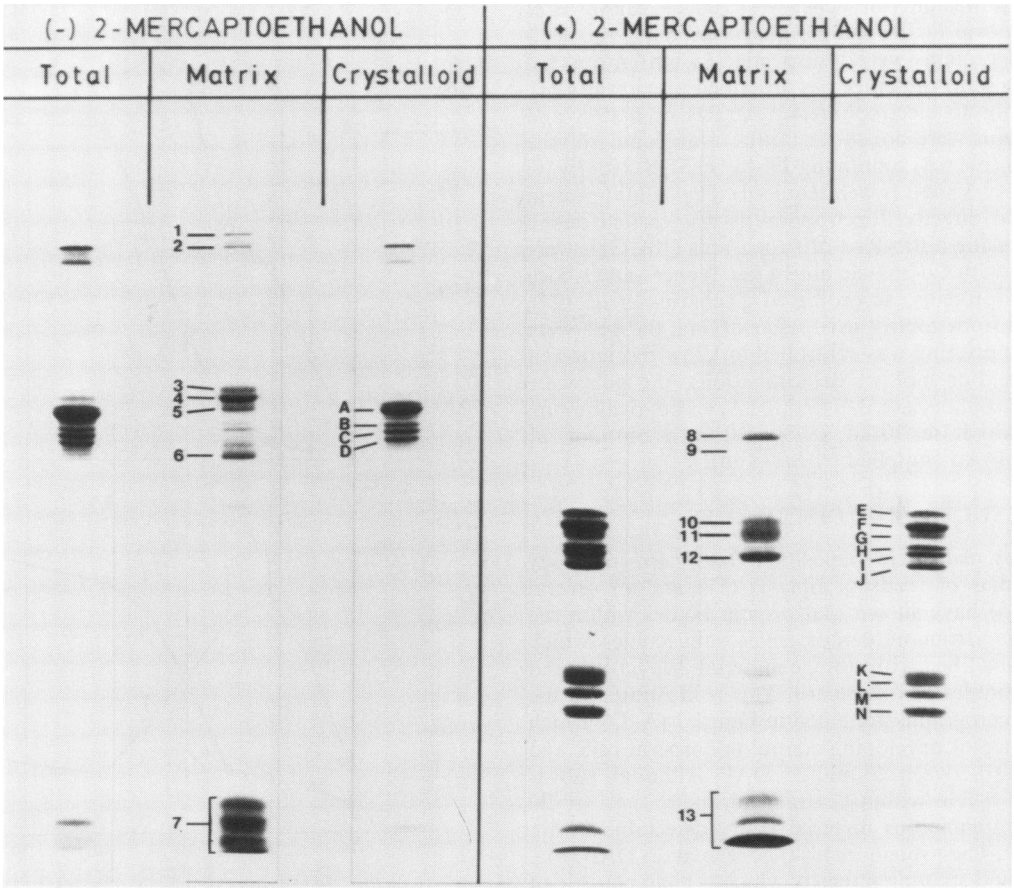

FIG. 3. SDS-PAGE profiles of matrix (M) and crystalloid (C) proteins extracted from protein bodies isolated from the endosperm of mature castor bean seeds. Profiles before and after ME treatment are shown, along with the band assignments used throughout the text. In each case, 30 μg of matrix and 10 μg of crystalloid protein in 10 μl of solubilizing buffer were applied to the gel.

Table I. *Approximate Molecular Weights of the Matrix and Crystalloid Proteins after SDS-PAGE*

The band assignments correspond to those shown in Figure 3 for specific matrix and crystalloid proteins from protein bodies of mature (60 DAP) castor bean seed before and after ME treatment. Approximate mol wt of each band was determined by using protein standards (see "Materials and Methods") concurrently on the same gel. Mol wt are expressed in kd.

Matrix Proteins				Crystalloid Proteins			
−ME		+ME		−ME		+ME	
band	*mol wt*	*band*	*mol wt*	*band*	*mol wt*	*band*	*mol wt*
1	>100	8	46.0	A	53.0	E	34.0
2	>100	9	45.0	B	51.0	F	33.0
3	63.0	10	34.0	C	50.0	G	32.0
4	60.0	11	33.0	D	49.0	H	31.0
5	56.0	12	30.0			I	30.0
6	45.0	13	<14			J	29.0
7	<14					K	23.0
						L	22.5
						M	22.0
						N	21.0

band 7. This latter group (band 7) corresponds to the matrix storage albumins described by Youle and Huang (22) and is further resolved on 15% gels into two major proteins with mol wt 12 and 10.5 kd (data not shown).

Previously (18), it has been shown that glycoproteins are present within the matrix of protein bodies from mature castor bean endosperm. To identify which of the bands contain carbohydrate moieties, the gels were examined after PAS staining, a procedure specific for glycoproteins (24). Figure 4A (60 DAP) clearly shows that the two groups represented by bands 1 and 2, and bands 3 and 4 are glycoproteins. These proteins correspond to RCA_I (bands 1 and 2) and ricin D (bands 3 and 4), which have been described in previous studies (13, 14, 18) of the protein body matrix from mature castor bean endosperm. Other than these two lectins, no storage proteins in protein bodies from mature castor bean endosperm are glycosylated.

After treatment with ME, three glycoprotein bands are evident (Fig. 4B). These are the corresponding subunits to the two lectins (13, 18); bands 10 and 12 are the 34 kd and 30 kd subunits of RCA_I, and bands 11 and 12 the 33 kd and 30 kd subunits of ricin D (Fig. 3).

While the gel profiles of matrix storage proteins from mature protein bodies are similar to those reported previously (18, 21, 22), the crystalloid protein profiles shown in Figure 3 are different and more complex. We suspected that limited proteolytic activity during the extraction procedure might account for the differences we observed, but the inclusion of PMSF (a potent inhibitor of proteolysis) during the extraction caused no alterations to the gel profiles.

The crystalloid proteins resolve into several bands on 12% gels (Fig. 3, bands A–D), which range in mol wt from 53 to 49 kd. These are reduced by ME treatment into two complementary groups (bands E–J and K–N), with respective mol wt ranging from 34 to 29 kd and 23 to 21 kd.

Changes in Polyacrylamide Gel Profiles. The extracts used to

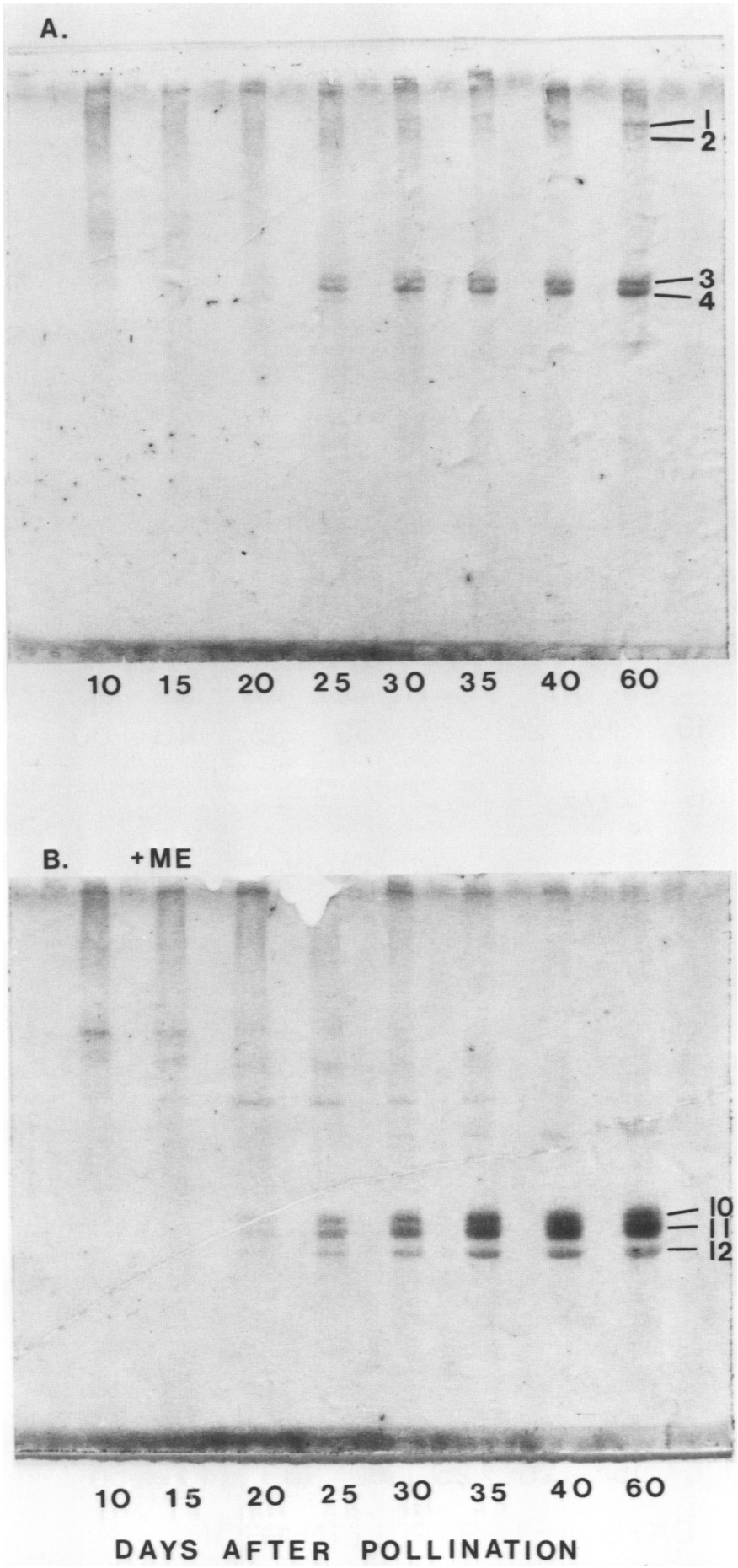

FIG. 4. SDS-PAGE profiles of extracts from whole endosperms of developing castor bean seeds after PAS staining, showing the glycoproteins within the total matrix and noncrystalloid fraction before (A) and after (B) ME treatment. Protein was applied to the gel in 10 μl, and concentration was based upon the total protein (mg/g dry weight) in the matrix and noncrystalloid fraction shown in Figure 2B, as follows: +ME, 75%, and −ME, 45%, of total protein at a given stage of seed development.

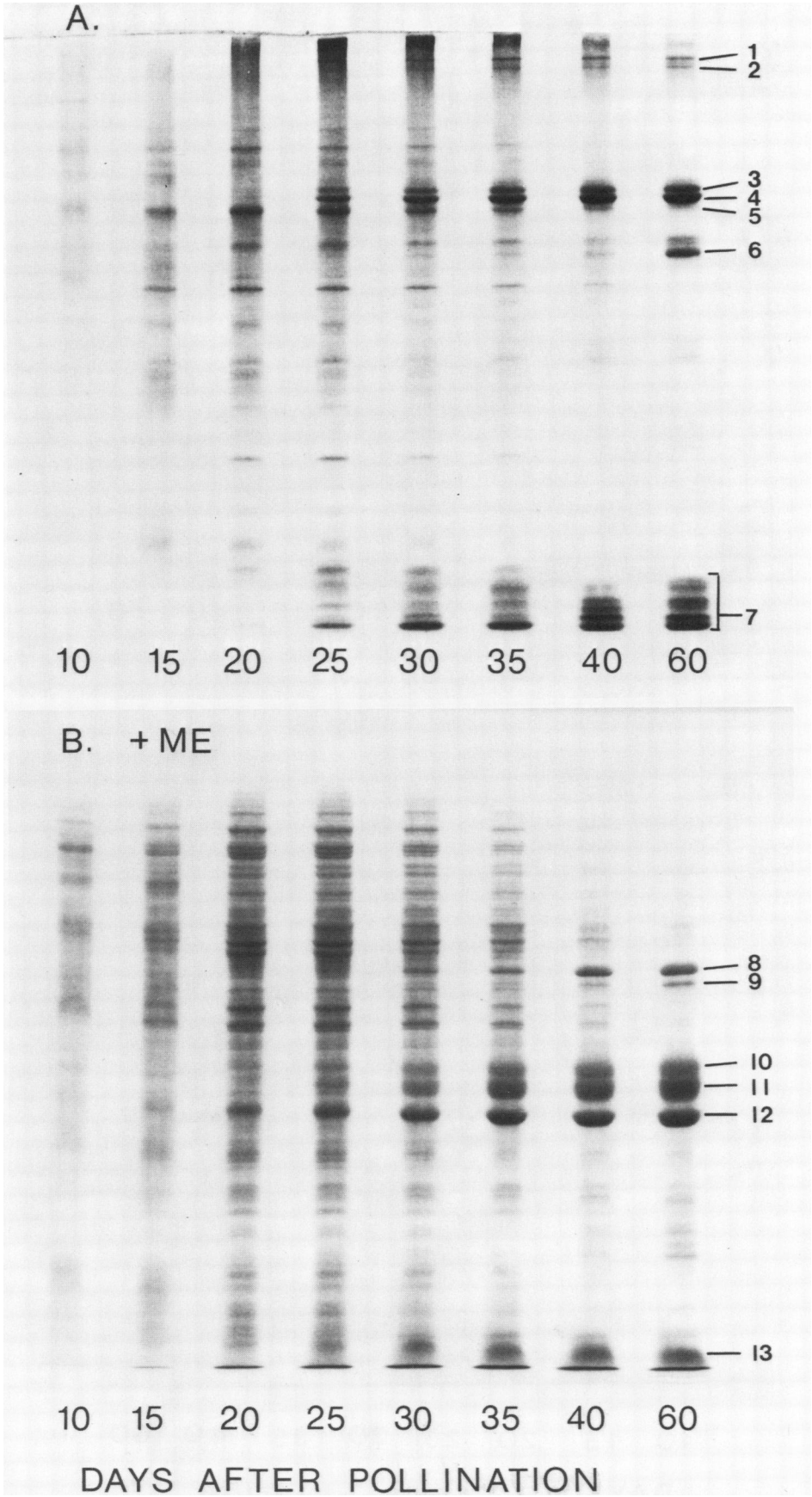

FIG. 5. SDS-PAGE profiles after Coomassie blue staining, showing protein changes within the matrix and noncrystalloid fraction before (A) and after (B) ME treatment. Profiles were obtained by using the same material and protein concentrations as those outlined in Figure 4.

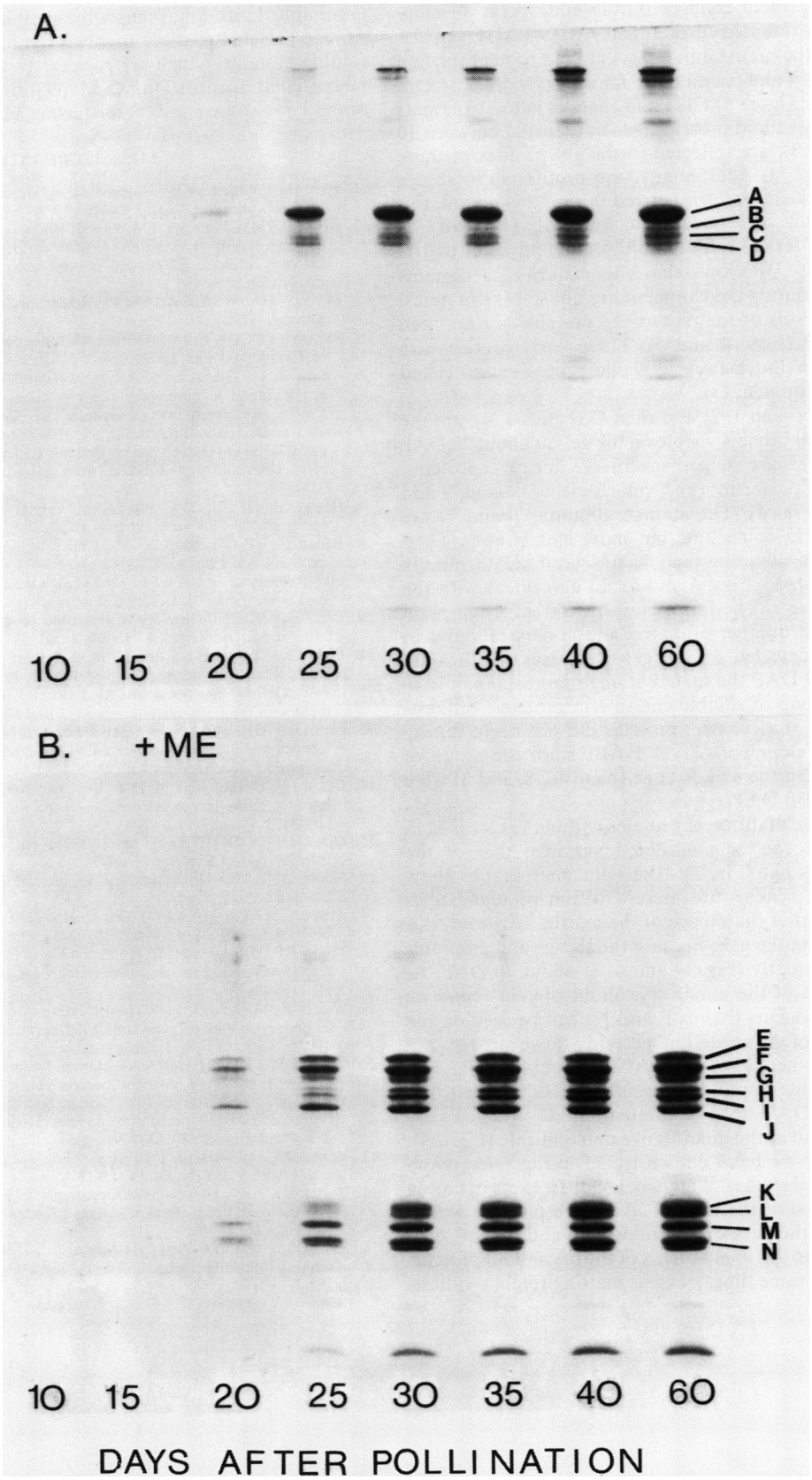

FIG. 6. SDS-PAGE profiles of extracts from whole endosperms of developing castor bean seeds after Coomassie blue staining, showing the crystalloid proteins before (A) and after (B) ME treatment. Protein was applied to the gel in 10 μl, and concentration was based upon the total protein (mg/g dry weight) within the crystalloid fraction shown in Figure 2A, as follows: +ME, 20%, and −ME, 10%, of the total protein at a given stage of seed development.

follow quantitative protein changes during endosperm development (Fig. 2) were examined further, using SDS-PAGE on 12% gels. The results for the matrix and noncrystalloid protein fraction are shown in Figures 4 and 5, and those for the crystalloid protein fraction are shown in Figure 6. The rapid changes in total amounts of matrix and noncrystalloid proteins, which occurred between 10 and 25 DAP (Fig. 2B), are reflected in the gel profiles of these protein extracts (Fig. 5). Additionally, the profiles show that a great diversity of proteins are synthesized during this period; this is particularly striking after treatment with ME (Fig. 5B). A comparison between these profiles and those obtained for matrix storage proteins (Fig. 3) shows that the majority of proteins appearing this early during development are not storage proteins. Indeed, detectable levels of matrix storage proteins are not seen until 20 to 25 DAP (Figs. 4 and 5). These early proteins are probably involved in the many metabolic processes associated with early seed development (3).

The lectins, RCA_I (band 1, 2) and ricin D (bands 3, 4), are the first matrix proteins to become visible on the gels at about 20 DAP (Fig. 5). When the gels are stained specifically for glycoproteins, the early synthesis of the lectins (particularly ricin D, bands 3 and 4) is obvious also (Fig. 4). The storage albumins (band 7) are detectable at about the same time but more clearly at 25 DAP (Fig. 5). The soluble albumins and lectins accumulate rapidly between 30 and 40 DAP (Figs. 4 and 5). In agreement with the quantitative data (Fig. 2B), during this period of endosperm development the total number of matrix and noncrystalloid proteins which can be detected on the gels decreases significantly (Fig. 5B), until by 40 DAP the matrix storage proteins (bands 8–13) are the major group of soluble endosperm proteins. Interestingly, the deposition of all matrix proteins did not begin during early endosperm development (20–25 DAP), since the group of proteins with mol wt in the 45 kd range (bands 6, 8, and 9) were not detected prior to 30 DAP.

While the deposition of different proteins within the castor bean protein body matrix can be considered variable, that of the crystalloid proteins is not (Fig. 6). The light micrographs show (Fig. 1A) that these proteins are present within protein bodies approximately 5 d earlier than those of the matrix. However, this is not so apparent from the gels, because the lectins and albumins are detectable concurrently (Figs. 4 and 6) at about 20 DAP. At this time, the subunit of the major crystalloid protein (band A) and its peptide components (bands F and N) can be seen on the gels. Major synthesis of the crystalloid proteins, however, does not begin until 25 DAP, when all of the crystalloid subunits (Fig. 6A) and their corresponding peptides (Fig. 6B) can be seen clearly. Between 25 and 40 DAP, their accumulation, as visualized by SDS-PAGE, is as rapid as the quantitative data indicate (Fig. 2A).

In summary, then, we have demonstrated, using micrograph and polyacrylamide gel studies, that there are differences in timing between the deposition of the crystalloid storage proteins within the protein bodies of the developing endosperm and that of some matrix storage proteins. All components of the crystalloid proteins are synthesized at the same time, whereas matrix protein synthesis is variable. In its initial stages, the synthesis of insoluble crystalloid storage proteins possibly involves the formation of many smaller soluble proteins, which are packaged subsequently in the protein body. Their identification and relationship to the proteins described above are subjects for further investigation.

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