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The Localization of Oat (*Avena sativa* L.) Seed Globulins in Protein Bodies

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ABSTRACT

The major storage proteins of the oat grain are the 12S and 7S globulins. Using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-diffusion assays we have demonstrated that the 7S globulin is localized predominantly in the embryo and the 12S globulin chiefly in the endosperm. Protein bodies have been isolated using aqueous and non-aqueous media and sucrose density gradient ultracentrifugation. Assaying marker enzymes gave results consistent with the presence of vacuolar protein bodies in the preparations. SDS-PAGE of sequential salt and alcohol extractions demonstrated the presence of globulins and prolamins respectively and their distribution within the gradient suggested that they may be localized in different protein bodies.

Key words: *Avena sativa* L.; Seed globulins; Protein bodies; Localization.

INTRODUCTION

In most cereal grains, the major seed storage proteins in the starchy endosperm are classified as prolamins, although the storage proteins extracted from the aleurone layers, scutellum and embryonic axis of wheat and barley have been shown to be globulins (Danielsson, 1949; Saverborn, Danielsson, and Svedberg, 1944).

In oats, the major storage proteins in the starchy endosperm are classified as globulins, with prolamins accounting for less than 15% of the total grain N (Peterson and Smith, 1976). The globulins have sedimentation coefficients ($S_{20\cdot w}$) of 12S and 7S (Peterson and Brinegar, 1983; Burgess, Shewry, Matlashewski, Altosaar, and Miflin, 1983), and certain other similarities to those present in the seeds of legumes and other dicotyledonous plants (Derbyshire, Wright, and Boulter, 1976; Shewry and Miflin, 1984). One of the characteristics of seed storage proteins is that they are deposited in protein bodies (Miflin and Shewry, 1979; Neumann and Weber, 1980). The development of these protein bodies in oilseeds (Dieckert and Dieckert, 1976) and legumes (Boulter, 1979; Bollini, Van der Wilden, and Chrispeels, 1982), is thought to involve transport of the proteins from the rough endoplasmic reticulum (RER), where they are synthesized, to be deposited in vacuoles. In barley, maize and wheat, however, the prolamin storage proteins are synthesized on the RER and then believed to aggregate into clumps within it (Larkins and Hurkman, 1978; Miflin, Burgess, and Shewry, 1981). Considerably less work has been done on the synthesis and deposition of non-prolamin proteins in cereals. Adeli, Allan-Wojtas, and Altosaar (1984) used pulse-labelling and found that the 12S globulin of oats was transported from the endoplasmic

reticulum and deposited in protein bodies. Saigo, Peterson, and Holy (1983) examined the ultrastructural development of the protein bodies in the starchy endosperm of oat and suggested that the storage protein is synthesized on membrane-bound ribosomes, transported within the ER cisternae, and deposited in vacuoles.

A variety of methods have been used to isolate globulin containing protein bodies from seeds; for example, Van der Wilden, Herman, and Chrispeels (1980) and Miflin *et al.* (1981) have used aqueous isolation followed by sucrose density gradient centrifugation to isolate legume seed protein bodies, whereas Yatsu and Jacks (1968) and Tully and Beevers (1977) extracted seeds in glycerol followed by differential centrifugation to produce pure protein body preparations from castor bean. This paper reports on the extraction of embryo and endosperm protein bodies of oats in aqueous and non-aqueous media and the localization of the major globulin proteins.

MATERIALS AND METHODS

Materials

Developing grains were harvested from oats (*Avena sativa* cv. Maris Tabard) grown in a greenhouse with supplemental light. Where embryos and endosperms were dissected from developing grain, cross contamination was checked by visual examination using a dissecting microscope at a $\times 10$ magnification.

Immunology

Oat globulins were prepared according to Burgess *et al.* (1983) and lyophilized proteins were resuspended for injection. A total globulin extract was dissolved in sodium phosphate pH 7·0 (3·0 mg in 1·0 cm^3) and a preparation of the large sub-unit of the 12S globulin was dissolved in 1% Triton X-100 in phosphate buffered saline pH 7·5 also at 3·0 mg in 1·0 cm^3. An enriched preparation of the 7S globulin obtained by sucrose density gradient centrifugation (Burgess *et al.*, 1983) was layered as a streak on a polyacrylamide slab gel and electrophoresed as for SDS-PAGE. The band corresponding to the major component of 7S globulin was excised, ground in 70% formic acid, then stirred for 1 h twice. The extractions were bulked and diluted to 1 in 10 with water, lyophilized and then resuspended in phosphate buffered saline (PBS) pH 7·5 for injection. The rabbits were injected intramuscularly in the leg with an emulsion of the protein in Freunds complete adjuvant. Blood was taken every 2 weeks and the serum separated from the cells after clotting. Protein fractions were assayed for activity with the antisera using immunodiffusion (Ouchterlony and Nilsson, 1973) in 1% agar in PBS containing 1000 mol m^{-3} NaCl. In some instances the plates were dried and stained in 0·09% amido black in acetic acid : methanol : water as 10 : 45 : 45 by vol. This solvent was also used as a destainer.

Isolation of protein bodies

(a) *Aqueous extraction*: Endosperms or embryos from developing seed were chopped with a razor blade in medium containing 400 mol m^{-3} sucrose, 1·0 mol m^{-3} EDTA, 0·1% bovine serum albumin, 10 mol m^{-3} potassium chloride, 0·1 mol m^{-3} magnesium chloride and 1·0 mol m^{-3} dithiothreitol in 50 mol m^{-3} Tris HCl pH 7·0. The tissue to medium ratio was 1 : 2·5–3·0 and the homogenate was filtered by squeezing through four layers of cheesecloth. The filtrate was centrifuged at 1000 *g* av. for 2 min at 4 °C to remove starch, and the supernatant was layered onto linear sucrose gradients (25%–65% w/w) in 50 mol m^{-3} Tricine pH 7·5 containing 100 mol m^{-3} potassium acetate and 1·0 mol m^{-3} EDTA and centrifuged at 70000 *g* av. for 2·5 h at 4 °C. The gradients were fractionated using an ISCO density gradient fractionator (model 640), and 1·2 cm^3 fractions collected.

The protein containing fractions of the gradients of endosperm extracts were determined by their absorbance. These fractions were halved at the middle fraction which corresponded to a density of 1·23 g cm^{-3} and the sucrose concentration of each reduced to 25% by gently adding 50 mol m^{-3} Tricine buffer pH 7·5. The resulting two fractions were re-centrifuged on linear sucrose density gradients (25% to 65% w/w) for 18 h, then fractionated as above.

(b) *Glycerol extraction*: Isolated endosperms or embryos from developing and mature seed were chopped with a razor blade in glycerol, at a ratio of 1 : 3 tissue to glycerol, until the material was finely divided. With older material the chopped tissue was then ground in a pestle and mortar to ensure complete extraction. The homogenate was filtered by squeezing through two layers of cheesecloth and

the filtrate layered onto 10 cm^3 of 20% sucrose dissolved in glycerol (density equivalent to 1·37 g cm^{-3}). This was centrifuged at 1100 *g* av. for 5 min to remove starch, and the supernatant re-centrifuged under the same conditions. The second supernatant was then centrifuged at 41 000 *g* av. for 1·5 h and the pellet washed twice in glycerol and re-centrifuged at 41 000 *g* av. for 1·5 h. All centrifugations were carried out in a Beckman L5-50 centrifuge at 10 °C.

Assays

Aliquots of fractions from the glycerol and aqueous preparations were assayed for the vacuolar marker enzymes *N*-acetylglucosaminidase and phosphodiesterase for 15–20 min according to Chrispeels and Boulter (1975). In one experiment NADH-cytochrome c reductase activity was measured (Tolbert, 1974). The sucrose concentration was determined using a direct reading refractometer. Protein was measured by the Lowry method after precipitation and washing with cold 10% (w/v) trichloroacetic acid (Miflin *et al.*, 1981).

Protein extraction

The extraction of globulins and prolamins was carried out essentially as described by Shewry, Field, Kirkman, Faulks, and Miflin (1980). Samples from the subcellular isolations and dissected material were shaken with 10–15 vols of 2000 mol m^{-3} NaCl (three times for 1 h each) in 100 mol m^{-3} Tris HCl pH 8·0, then with distilled water for 1 h. The resulting supernatants were bulked, dialysed against distilled water for 24 h and lyophilized. The salt-insoluble pellets were shaken with 10–15 vols of 50% (v/v) propan-2-ol + 2% 2-mercaptoethanol (three times for 1 h each). The supernatants from this extraction were also bulked, dialysed against water for 24 h and lyophilized prior to separation by SDS-PAGE.

Electrophoresis

Samples were dissolved in 8000 mol m^{-3} urea, 65 mol m^{-3} Tris HCl pH 6·8, 4% SDS; 2% 2-mercaptoethanol and subjected to SDS-PAGE at pH 8·8 using a modified Laemmli gel system (Forde, Kreis, Bahramian, Matthews, Miflin, Thompson, Bartels, and Flavell, 1981). The gels were fixed and stained as described previously (Burgess *et al.*, 1983).

RESULTS

Antisera were obtained from rabbits injected with a total oat globulin fraction, 7S globulin and the large sub-unit of the 12S globulin. All of the antisera reacted with the total oat globulin protein, and similarly the total serum reacted with all of the oat antigens (Fig. 1a–c).

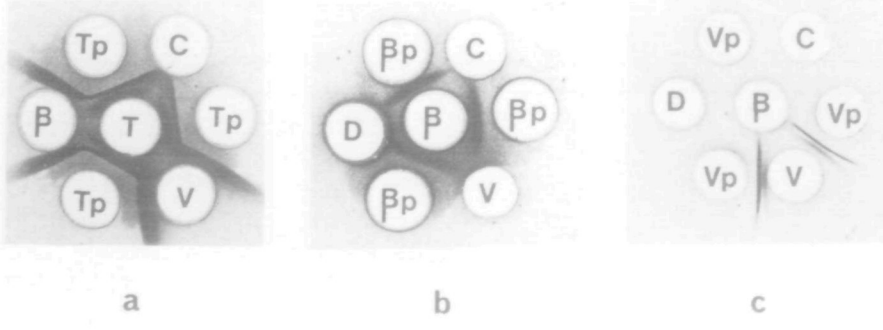

FIG. 1. Immunodiffusion plate analysis of globulins against specific antisera stained with amido black. The antigens are (a) the oat total globulin (Tp); (b) the large subunit globulin (Bp); (c) the 7S globulin (Vp). The antisera in adjacent wells were produced on injection with the following: C—non-immune serum; β—large subunit oat 12S globulin; D—12S oat globulin; T—total oat globulin; V—7S oat globulin. The serum to the 12S oat globulin (D) was subsequently discarded as no precipitin was produced in response to any antigen.

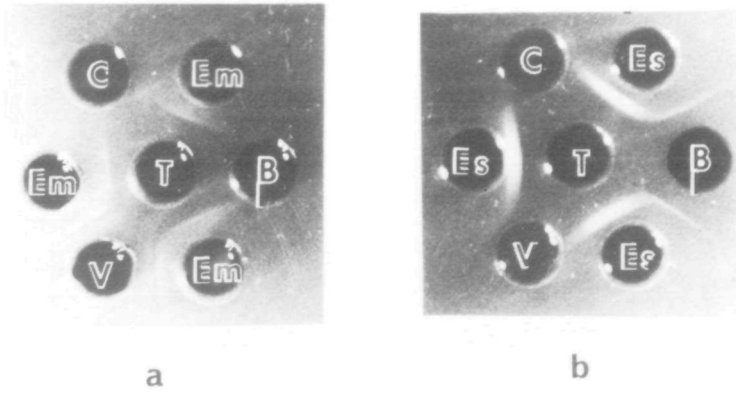

FIG. 2. Immunodiffusion plate analysis of globulins from dissected oat (a) embryo and (b) endosperm tissue. Em—embryo antigen; Es—endosperm antigen; C—non-immune serum; T—total antiserum; β—large subunit antiserum; V—7S antiserum.

No precipitin line was formed in response to the non-immune serum. Two bands developed with the 7S antiserum against the 7S protein which may indicate two antigenic determinants (Fig. 1c).

The globulins were isolated from embryos and endosperm separately. These were tested using immunodiffusion in agar plates with specific antisera for the presence of 7S and 12S globulins (Fig. 2). There was no response with either of the globulin preparations against the pre-immune serum, and both antigens gave a positive response with the total oat globulin antiserum. The embryo extract was reacted with the antiserum raised against the large sub-unit of the 12S globulin but no precipitin band formed, whereas two distinct precipitin bands developed against the 7S anti-serum (cf. Fig. 1c and Fig. 2a). When the endosperm extract was tested, the results obtained were reversed; a precipitin band was formed in response to the large sub-unit anti-serum and not to the 7S anti-serum (Fig. 2b).

Protein body isolation

Protein bodies were extracted from dissected embryos of oat grain using aqueous and glycerol extraction media. The aqueous extraction of these embryos gave a lower yield of protein bodies, but the same protein bands were present in the aqueous (results not shown) and the glycerol extraction (Fig. 3c). There is a major band of M_r 50 000 to 55 000 and a number of minor bands including a group around 59 000 to 65 000 (Fig. 3c). The band of M_r 50 000 to 55 000 corresponds to the 7S globulin previously described by Burgess *et al.* (1983). The globulin was also extracted directly from dissected embryos with 1000 mol m^{-3} NaCl, and analysed by SDS-PAGE (Fig. 3a), giving the same results as the glycerol protein body extraction (Fig. 3c). In both cases there is also a minor band corresponding to the large subunit of the 12S globulin of M_r 35 000 to 42 000.

SDS-PAGE of endosperm protein bodies isolated with glycerol is shown in Fig. 3d. The two most intensely-stained groups were between M_r 19 000 and 25 000 and M_r 35 000 and 42 000. These correspond to the subunits of the 12S globulin described by Peterson (1978) and Burgess *et al.* (1983). The results are similar to those obtained by aqueous extraction of

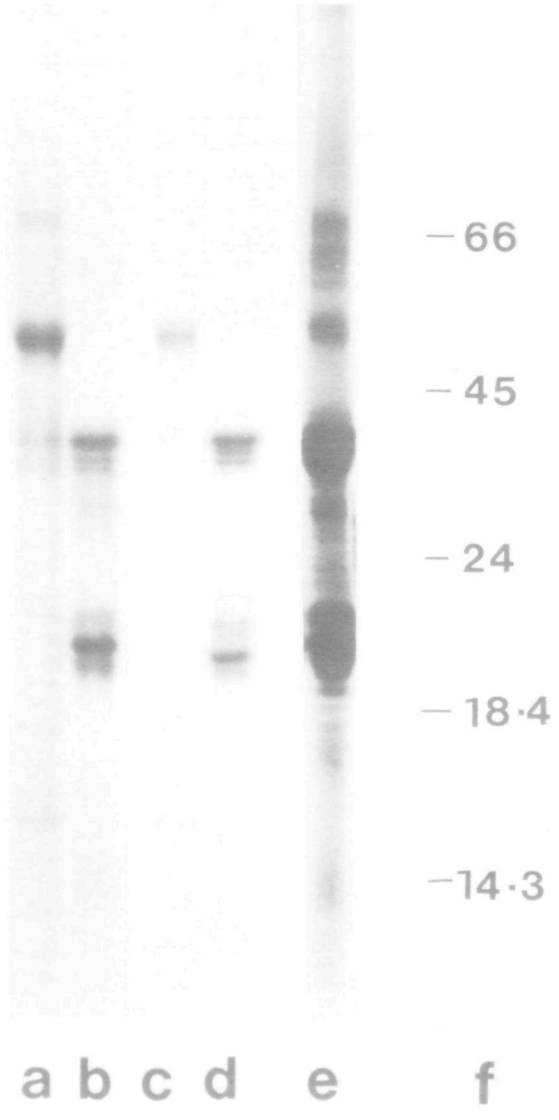

FIG. 3. Reduced SDS-PAGE of oat globulins from (a) hand-dissected embryos; (b) hand-dissected endosperms; (c) protein bodies of embryos prepared using glycerol; (d) protein bodies of endosperm as in (c); (e) total globulin from whole grain; (f) the apparent M_r was determined using the following mol. wt. markers; bovine serum albumin $M_r = 66\,000$; ovalbumin $M_r = 45\,000$; trypsinogen $M_r = 24\,000$; β-lactoglobulin $M_r = 18\,400$; lysozyme $M_r = 14\,300$. Their mobility is indicated on the gels by the M_r values ($\times 10^{-3}$).

dissected endosperm (Fig. 3b). Vacuolar marker enzyme activity was also found to be associated with the protein body fraction with a conversion rate for phosphodiesterase and *N*-acetyl glucosaminidase of about 6 nmol substrate converted min^{-1} mg^{-1} protein. This value is similar to the specific activities (4–7 nmol min^{-1} mg^{-1}) obtained from the peak protein body fractions on the sucrose gradients of aqueous homogenates of endosperms

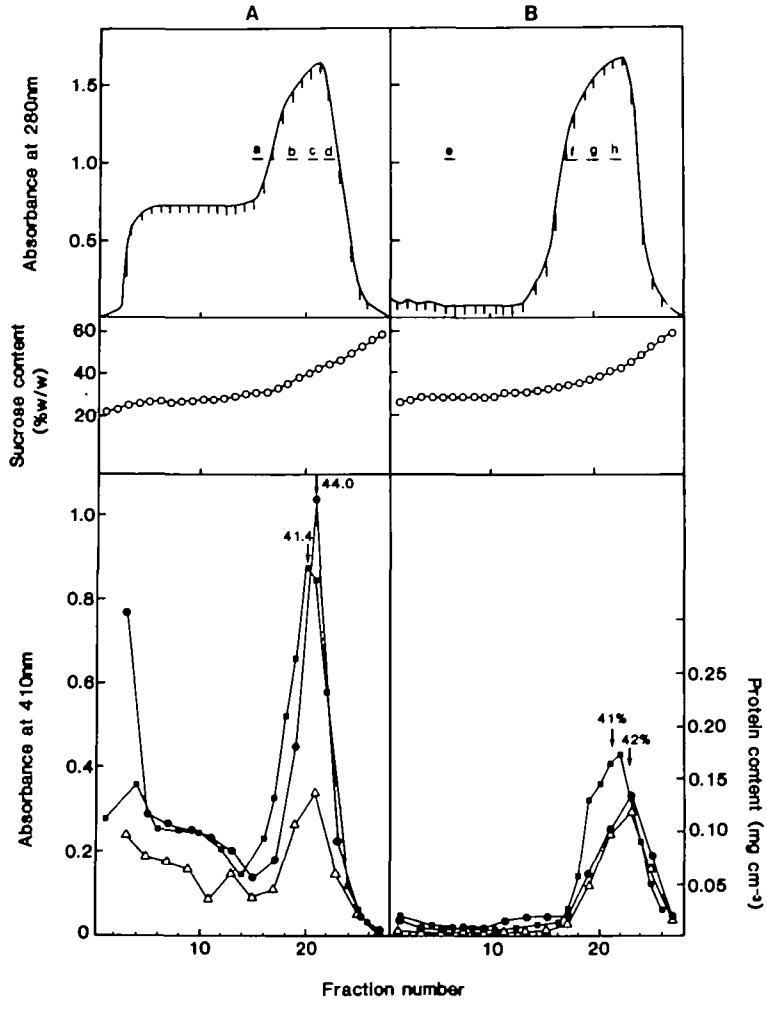

FIG. 4. Distribution of various components after sucrose gradient centrifugation. The peak of absorbance at 280 nm obtained from one sucrose gradient was split at a density of 1·23 g cm^{-3}. (A) The sucrose gradient of the material from 1·174 to 1·23 g cm^{-3}; (B) the sucrose gradient separation of the material from 1·23 to 1·275 g cm^{-3}. SDS-PAGE of the protein extractions of areas a–d and e–h are shown in Fig. 5. Enzyme assays of the sucrose gradients. ■—■ Phosphodiesterase; o—o *N*-acetyl glucosaminadase; ▲—▲ protein estimation.

(Fig. 4). The vacuolar enzyme activity remained with the protein body pellet after washing several times with the glycerol extraction medium.

Aqueous homogenates of developing endosperms containing protein bodies were subjected to sucrose density gradient centrifugation. A broad peak of material absorbing at 280 nm (results not shown) was obtained, distributed over a density of 1·174 to 1·275 g cm^{-3}. This material was split at a density of about 1·23 g cm^{-3} and the sucrose concentration of both fractions reduced to 1·107 g cm^{-3} (equivalent to 25% w/w sucrose) by gently adding gradient buffer. The two were then recentrifuged on sucrose density gradients and fractionated, and the absorption at 280 nm continuously monitored (Fig. 4A, B). The activities of *N*-acetyl glucosaminidase and phosphodiesterase, both vacuolar marker enzymes, were also measured (Fig. 4A, B), and the maximum activity of the enzymes is in the same area of the

gradient as the maximum protein content. For both the gradients (Fig. 4A, B), the maximum activity of *N*-acetyl glucosaminidase is at a slightly higher density compared to phosphodiesterase activity.

Gradient fractions were extracted for globulins and prolamins and subjected to SDS-PAGE (Fig. 5). The major large and small sub-units of the 12S globulin, of M_r 34 000 to 42 000 and M_r 19 000 to 25 000 respectively, were present in both of the gradients. Figure 5A, a–d shows SDS-PAGE of the globulin extraction from the gradient shown in Fig. 4A, and Fig. 5A e–h the extraction from the gradient shown in Fig. 4B. Equal volumes were extracted but more globulin is present on the gradient corresponding to the less dense region of the original sucrose density gradient (i.e. Fig. 4A), although all of the globulin containing fractions in Fig. 5A correspond to a density of 1·19 to 1·20 g cm^{-3}. SDS-PAGE of the prolamins extracted from the sucrose density gradients is shown in Fig. 5B with an oat prolamin standard. Figure 5B a–d relates to the absorbance trace shown in Fig. 4A and Fig. 5B e–h to the absorbance trace shown in Fig. 4B. A group of bands of M_r 58 000 to 62 000 was present, see particularly track b of Fig. 5B, which is from a density of 1·16 g cm^{-3}. The other major bands present are equivalent to those in the standard oat prolamin, mainly in the region M_r 22 000 to 35 000. In contrast to the globulin, more prolamin was extracted from the gradient derived from the more dense region of the original sucrose gradient. The prolamin was extracted from gradient fractions having a density of 1·20 to 1·23 g cm^{-3}: a greater density than for the major globulin-containing fractions.

DISCUSSION

The 12S and 7S globulins are the major storage proteins of the oat grain with the 12S being in greater abundance (Peterson, 1978; Burgess *et al.*, 1983). The location of these components within the grain was first discussed by Osborne (1924). Subsequently Saverborn *et al.* (1944), Danielsson (1949) and Morita and Yoshida (1968) showed that the 7S (or γ) globulins of wheat, barley and rice, respectively, were located mainly in the embryo; they did not investigate the location of the 12S globulin. The results in this study, based on the hand dissection of grain followed by total globulin extraction and electrophoresis, and the isolation of protein bodies from the separate tissues, confirms that the 7S proteins are predominantly in the embryo and the 12S in the endosperm.

The globulins in these tissues are clearly located within protein bodies. These protein bodies have many properties in common with those isolated from legumes (Van der Wilden *et al.*, 1980; Burgess *et al.*, 1983), particularly with respect to the presence of vacuolar marker enzymes. Attempts to separate the globulin-containing bodies from the prolamin-containing bodies in the endosperm were not completely successful. However, the density of the fractions for the peak of prolamin-containing bodies differed slightly from those for the globulins (1·21–1·23 g cm^{-3} compared with 1·17 g cm^{-3} see Figs 4 and 5) suggesting the presence of two populations of protein bodies. Yamagata, Tanaka, and Kasai (1982) working with rice have been able to separate the glutelin-containing protein bodies from the prolamin-containing protein bodies; this is of relevance because rice glutelins are homologous with oat globulins (Zhao, Gatehouse, and Boulter, 1983). The oat protein bodies are fragile which hampers their isolation; resolution of the location of the storage proteins may need to be determined using an immunological approach coupled to light and/or electron microscopy.

The differential location of 7S and 12S globulins in oat grain contrasts with the findings in legumes where both proteins are found predominantly in the cotyledons (Boulter, 1979). Using immunocytochemistry with fluorescent antibodies it has been shown that the 7S and 12S globulins are present in the same protein bodies (Craig, Goodchild, and Millerd, 1979).

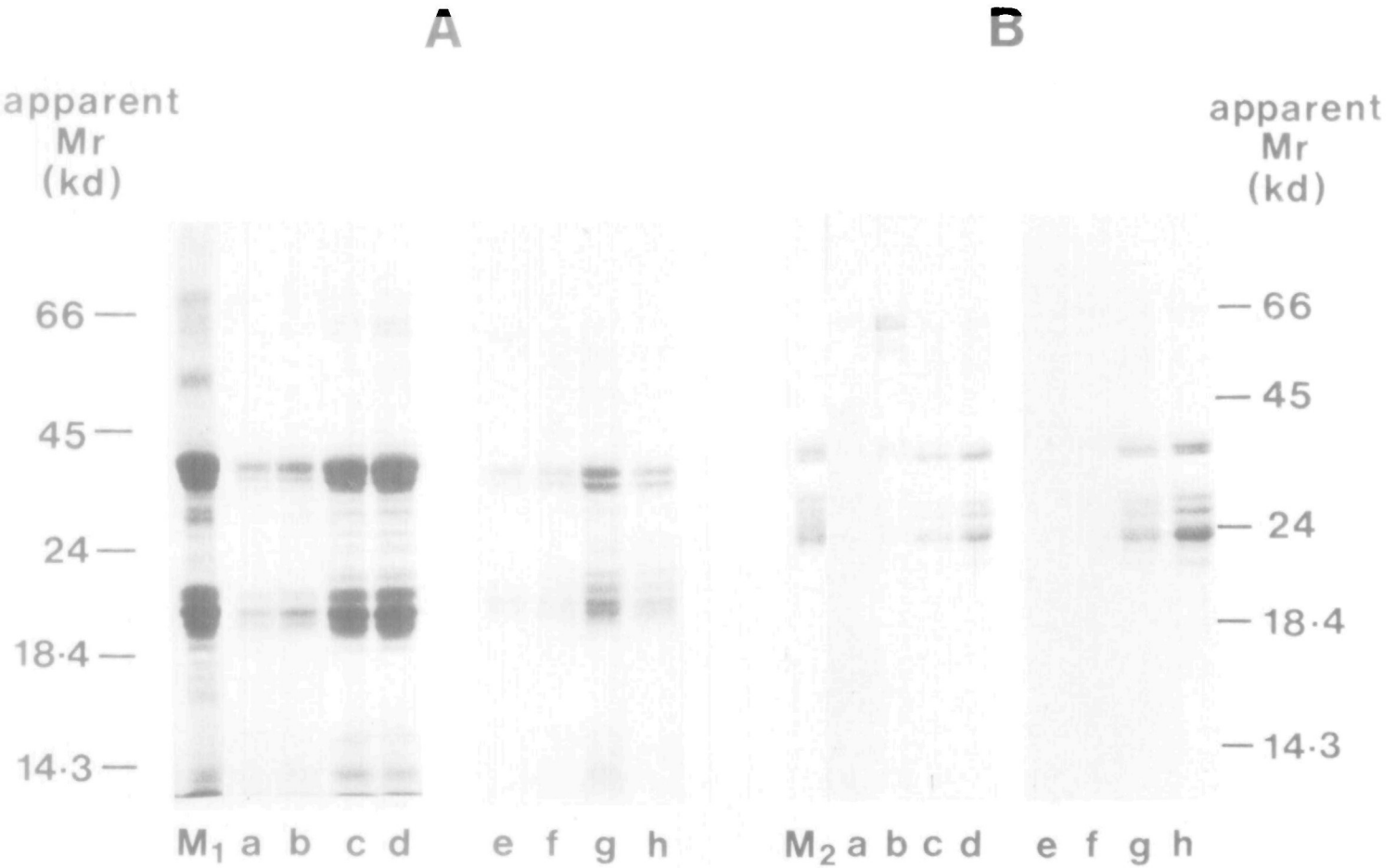

FIG. 5. Reduced SDS-PAGE of globulin and prolamin extractions of fractions from the sucrose density gradients. (A) Globulin extracted with 2000 mol m^{-3} NaCl; (B) prolamin extracted with 50% (v/v) propan-2-ol and 2% (v/v) 2-mercaptoethanol. M_1 is a standard of oat globulin. M_2 is a standard of oat prolamin. Areas a–d and e–h correspond to the areas of the sucrose density gradient shown in Fig. 4A and B respectively. The apparent M_r was determined using the following mol. wt. markers; bovine serum albumin M_r = 66 000; ovalbumin M_r = 45 000; trypsinogen M_r = 24 000; β-lactoglobulin M_r = 18 400; lysozyme M_r = 14 300.

The reasons for the differential expression of the globulin storage proteins, both in terms of amounts and location, in different cereals and legumes may be determined by further isolation and detailed comparisons of the genes from the different species.

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