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Biochemistry of Fern Spore Germination: Globulin Storage Proteins in *Matteuccia struthiopteris* L.[1]

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ABSTRACT

Two globulin storage proteins have been identified in spores of the ostrich fern, *Matteuccia struthiopteris* (L.) Todaro. The two proteins comprise a significant amount of the total spore protein, are predominantly salt-soluble, and can be extracted by other solvents to a limited extent. The large 11.3 Svedberg unit (S) globulin is composed of five polypeptides with molecular weights of 21,000, 22,000, 24,000, 28,000 and 30,000. Each polypeptide has several isoelectric point (pI) variants between pH 5 and 7. The small 2.2S storage protein has a pI > 10.5 and is composed of at least two major polypeptides of 6,000 and 14,000 M_r. The amino acid composition of both storage proteins reveals that the 11.3S protein is particularly rich in aspartic and glutamic acid, while the 2.2S protein has few acidic amino acids. During imbibition and germination the globulin fraction declines rapidly, with a corresponding degradation of individual polypeptides of each protein. Polyclonal antibodies against each of the two proteins were produced and used for immunolocalization to determine the site of storage protein deposition within the quiescent spore. The proteins were sequestered in protein bodies of 2 to 10 micrometers, that are morphologically similar to those found in the seeds of flowering plants. The results suggest that spore globulins are biochemically similar to seed globulins, especially those found in some cruciferous seeds.

Increased attention is being given to the chemical and genetic characterization of seed storage proteins. They are present in significant quantities in mature seeds and they provide a readily available source of carbon and nitrogen for synthetic processes occurring during seedling growth (14). Globulins, or salt-soluble proteins, are the major storage proteins of dicotyledons, while monocotyledons usually contain protein reserves soluble either in aqueous alcohol or in dilute acids or alkali (prolamins and glutelins, respectively). There are numerous exceptions to this general distribution of storage proteins, and it is now recognized that the various classes of proteins are not restricted to specific groups of plants. Investigators have demonstrated that significant homology exists between the storage proteins of evolutionarily distinct angiosperms (17, 25). It has been suggested that homologous sequences evolved from a common ancestor and were conserved during evolution. Most analyses have been directed to the storage proteins of agriculturally important plants and very little research has been done with proteins of phylogenetically primitive plants. This work is required in order to elucidate the evolution of these important storage compounds.

Some pertinent information concerning storage proteins in the spores of bacteria and algae is available. Bacteria contain storage proteins that accumulate in large quantities during spore development (26). In *Anabaena cylindrica*, a cyanobacterium, a storage polypeptide has been identified that is sequestered during spore formation and stationary phase cultures (27, 28). In both cases, the function of the storage proteins is similar to that of seeds, providing a readily accessible source of nitrogen upon the resumption of growth.

Lower vascular plants also have been shown to contain storage proteins. Reports have described storage protein decline in fern spores during imbibition and germination (22, 24). However, since no fern spore storage proteins have been isolated and characterized, the developmental role of these proteins remains unclear.

Gymnosperms, a group that contains many higher vascular plants of ancient lineage, contain seed storage proteins within haploid megagametophyte tissue. While investigators have identified storage proteins in various pine species (13, 18), only in the cycad, *Macrozamia communis*, has gymnosperm storage protein been isolated and partially characterized (1).

Clearly more work is required with primitive plant groups in order to determine whether the formation of ancestral storage protein genes occurred before higher vascular plants arose. This paper describes research concerned with the identification, partial characterization, and localization of the principal storage proteins in the spores of the ostrich fern, *Matteuccia struthiopteris*. The qualitative and quantitative data presented here indicate that fern spores have protein bodies containing globulin storage proteins that are biochemically similar to certain angiosperm seed storage proteins.

MATERIALS AND METHODS

Plant Material. Mature fertile leaves of *Matteuccia struthiopteris* (L.) Todaro were collected from several locations in Hanover, NH, in late autumn and spores were released by repeatedly soaking the leaves in water and then drying them at room temperature. Spores were separated from associated organic debris using an Allen Bradley sonic sifter and stored in dark bottles at 4°C. For germination time course studies, 200 mg spores were placed in 100 ml of distilled H_2O in a 1-L Erlenmeyer flask and maintained on a 12 h light-dark cycle at 25°C in a growth chamber. Illumination (250 ft-c = 40 μE m^{-2} s^{-1} PAR) was provided by an equal ratio of cool fluorescent to incandescent lamps.

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Protein Extraction and Determination. Two g of spores were ground in 2 ml of distilled H_2O using a motorized glass tissue grinder (Duall 25, Kontes) held on ice until greater than 90% of the spores were broken, as determined by microscopic observation. Proteins were extracted and fractionated into various solubility classes following a modification of the procedure described by Hu and Esen (15), outlined in Figure 1. TCA at a final concentration of 10% was added to each fraction and the material was chilled in an ice bath for 15 min to precipitate protein. Following centrifugation at 2700*g* for 15 min, the precipitate was washed repeatedly with cold acetone to remove Chl. The pellet was dissolved in 5 ml of 2 N NaOH, and soluble protein was determined along with total spore protein according to Spudich and Kornberg (29).

Sucrose Gradients and Native Gel Electrophoresis. Sedimentation equilibrium analysis of the globulin proteins was accomplished on 10 to 30% sucrose gradients spun in a Beckman SW27 rotor at 22,000*g* for 24 h at 15°C. Soybean trypsin inhibitor (11.3S), yeast alcohol dehydrogenase (7.2S), and bovine liver catalase (2.2S) were used as calibration standards. A calibration tube containing 10 mg of each standard was companion-run in each centrifugation.

Native gel electrophoresis (30), was performed on the large spore storage protein to confirm the S value obtained from the sucrose gradients. Ferguson plots were constructed and the mol wt of the 11.3S protein was determined (Sigma, technical bulletin MKR-137).

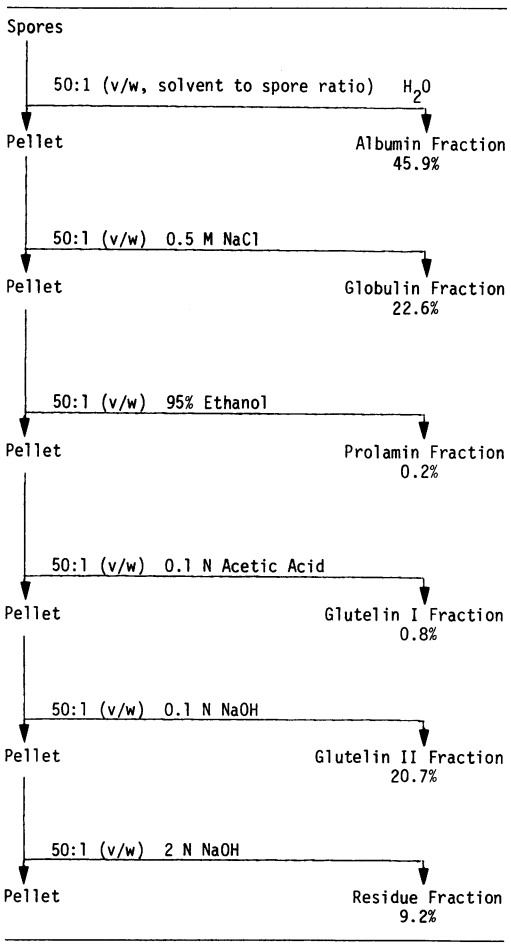

FIG. 1. Flow diagram of the protocol used for solubility fractionation of fern spore proteins and for determining the percent of total protein soluble in each fraction. Albumins and globulins were extracted at 4°C, with all other extractions done at room temperature (22–23°C). Each extract was agitated for 1 h, before centrifugation at 2700*g* at 5°C for 15 min.

Polyacrylamide Gel Electrophoresis. For protein band analysis by SDS-PAGE in one dimension, the globulin fraction was precipitated by dialysis against distilled water overnight, collected by centrifugation at 2700*g* for 15 min, and dissolved in running buffer (0.19 M glycine, 0.1% SDS, 25 mM Tris, pH 8.3). Approximately 45 mg of protein was loaded in each gel lane, except where noted. Polypeptide separation was achieved on 5 to 15% gradient gels and the gels were stained with CBB. Mol wt standards (14–66,000) were obtained from Sigma. To resolve the smaller polypeptides of the 2.2S protein in one dimension, a 15% urea gel was employed as described by BRL (2). Low mol wt standards (3–43,000) were included and gels were silver-stained.

Two-dimensional polyacrylamide gel electrophoresis was done according to the procedure of O'Farrell (23). Ampholytes (LKB) with a pH range of 3.5 to 10 and 5 to 7 were used in isoelectrofocusing gels in a ratio of 1:4, respectively. To achieve polypeptide separation, a 5 to 15% gradient was used in the second dimension.

Amino Acid Analysis. Approximately 1 nM of each of the two spore storage proteins was hydrolyzed in 6 M HCl for 24 h at 110°C in sealed tubes. The acid was removed by evaporation in a vacuum desiccator and the hydrolysate was analyzed in a Beckman 121-MB amino acid analyzer (Protein Chemistry Laboratory, Harvard University).

Preparation of Antisera. The total globulin fraction from *M. struthiopteris* and each of the two proteins (2.2S and 11.3S) that comprise this fraction were isolated as described and separately injected intradermally into New Zealand white rabbits after collection of an aliquot of preimmune serum. The antigens (2 mg) were emulsified with an equal amount of Freund's complete adjuvant (Sigma). Booster shots consisting of the same mixture were given at 14 and 28 d. Ten d after the last injection, blood was removed from the rabbit and the serum was collected after blood clotting. Titer levels and antiserum specificity were checked by double diffusion in agarose gels. The serum was stored at −70°C, and thawed before use.

Immunolocalization of Storage Proteins. To determine the location of the ostrich fern storage proteins within the spore, thick sections were prepared and processed by a modification of the procedure of Craig and Goodchild (4). Ostrich fern spores were fixed at a ratio of 1:20 (w/v) for 12 to 24 h in a solution containing 15% paraformaldehyde, 2% glutaraldehyde, and 3% Triton X-100 in 50 mM cacodylate buffer (pH 7.0). The spores were washed three to four times in cacodylate buffer by low speed clinical centrifugation. Washed spores were embedded in 1% agar and the agar was cut into 1 mm cubes. The cubes were dehydrated through an alcohol series and infiltrated with Spurr's resin. Thick sections of 2 μm were cut, placed upon slides and allowed to dry.

To remove trace resin contamination from the sections, all slides were dipped in 100% ethanol for 30 s and rinsed in PBS-ST.[3] The spore sections were then incubated for 30 min in antiserum diluted 1:50, followed by a 30 min incubation in FITC-labeled, affinity-purified goat anti-rabbit second antibody (Miles) diluted 1:32. After each incubation, the spores were washed three times in PBS-ST. To minimize bleaching of the fluorescent signal during observation, coverslips were mounted in 0.1% phenylenediamine in buffered glycerol at pH 8.0. Spore sections were viewed on a Nikon Optiphot epifluorescence mi-

[3] Abbreviations: PBS-ST, phosphate-buffered saline containing 500 mM NaCl and 1% Tween 20, pH 7.2; CBB, Coomassie brilliant blue; FITC, fluorescein isothiocyanate; IEF, isoelectric focusing; pI, isoelectric point.

croscope equipped with an excitation filter cassette B using a Zeiss 63×, 1.4 NA oil immersion objective. Immunofluorescence was minimized when colored filters were eliminated. Light micrographs were taken either with Kodak Tri-X film (developed in Kodak HB-110) or Kodak Ektachrome slide film.

RESULTS

Identification of Ostrich Fern Storage Proteins. Approximately 20% of the spore weight was determined to be protein. To identify the major storage proteins, the total protein was fractionated into various solubility classes (Fig. 1) and the amount of each protein class was determined. The percentages given in Figure 1 represent amounts of protein contained within the quiescent spore.

Within minutes of being placed in water, the spores swelled to an elliptical ovoid shape. Germination, as determined by protrusion of the rhizoid, was observed by d 2 (Fig. 2). The 3rd, 4th, and 5th d were marked by extensive rhizoid elongation and prothallial growth. By the 6th d, the fern prothalli had developed into 3-celled filaments.

Globulin protein rapidly decreased as the spores hydrated and the spore coat ruptured (Fig. 2). In the 1st d, 20% of the protein was degraded and by the 3rd d, 60% of the protein had been hydrolyzed. Most of the storage globulin was hydrolyzed during imbibition and the initial stages of germination and gametophyte development. An additional 20% decline in the globulin fraction occurred between d 3 and 6, when the gametophyte was undergoing rapid elongation, prior to the initiation of two-dimensional growth. No globulin protein was detectable once gametophytes had sexually matured, nor was globulin protein found in any of the sporophytic stages.

Sucrose density gradient analysis of the globulin protein isolated from spores prior to imbibition revealed two distinct peaks (Fig. 3). These were determined to have $s_{20,w}$ values of 11.3S and 2.2S. A value of 11.3S is comparable to a mol wt of 232,000 as determined from Ferguson plots of nondenaturing gels (Figs. 4 and 5). The 2.2S protein would not enter the nondenaturing gels, indicating that it was still positively charged at pH 8.4. Peak fractions were analyzed on 5 to 15% SDS-PAGE gradient gels. The 11.3S protein consists of two major bands with mol wt of 21,000 and 30,000, and three minor bands with mol wt of 22,000, 24,000, and 28,000 (Fig. 6A). The 2.2S protein consists of only one band whose mol wt is less than 14,000 on a 5 to 15% gel (Fig. 6A); however, when this protein was separated on a 15% urea gel, two bands of 6,000 and 14,000 were observed (Fig. 6B).

Time course analyses were carried out to determine the fate of

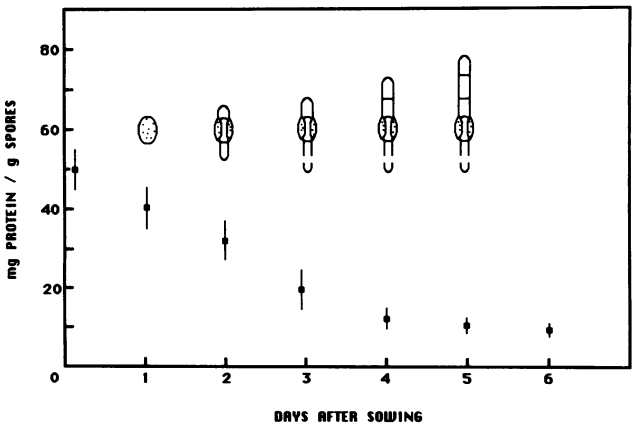

FIG. 2. Time course of changes in globulin protein levels during spore imbibition and germination. Each point represents the average of four determinations ± SD. The developmental stage of the spores is shown along the top of figure.

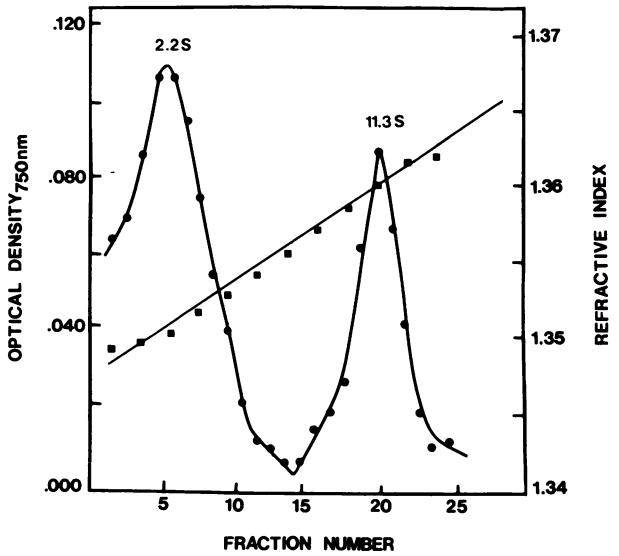

FIG. 3. Separation of ostrich fern storage globulins according to their sedimentation velocity on 10 to 30% sucrose gradients. The optical densities (●) are based on Lowry absorbance of 10 μl samples. Refractive indices (■) were determined for odd numbered fractions. The calibration standards sedimented in tube 5 (bovine liver catalase), tube 15 (yeast alcohol dehydrogenase), and tube 20 (soybean trypsin inhibitor).

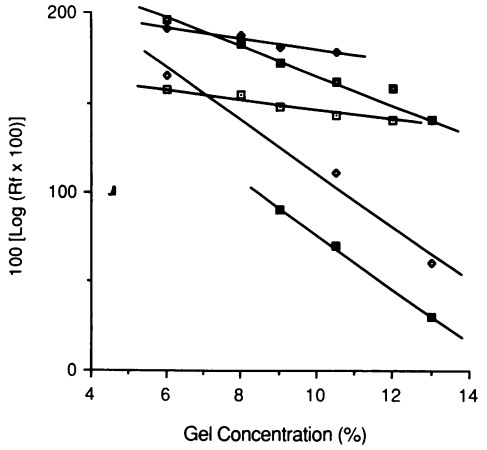

FIG. 4. Ferguson plot of the 11.3S spore storage protein (■) and the protein calibration standards: α-lactalbumin (◆), carbonic anhydrase (⊡), BSA (■), and urease (◇).

the individual globulin bands (Fig. 7). The globulins were separated on 5 to 15% SDS-PAGE gradient gels and bands were observed at 30,000, 28,000, 24,000, 22,000, 21,000 and <14,000. All of the bands exhibited equal degradation during the first 3 d after spore sowing and very little remained on the 6th d. The lower band (<14,000), which represents the polypeptides of the 2.2S protein, exhibited a much slower rate of degradation and was still observed on d 6.

Isoelectric Focusing and Two-Dimensional Polyacrylamide Gel Electrophoresis Analysis. A two-dimensional gel of the 11.3S protein is shown in Figure 8. Each of the bands observed in one-dimensional gels can be separated clearly into several polypeptides with different isoelectric points. There are at least 13 major peptides of 21,000 to 30,000 that can be distinguished within a 5 to 7 pI range, with the larger mol wt peptides being less acidic than the smaller peptides. IEF was attempted on the 2.2S protein, but the protein would not enter even extremely basic gels (pH 10.5). We concluded that the 2.2S protein has a pI of > 10.5.

Amino Acid Composition of the 2.2S and 11.3S Proteins. The

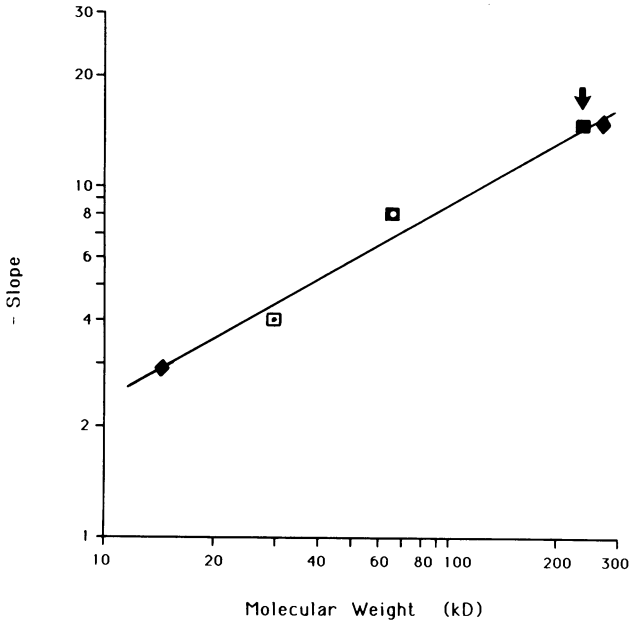

FIG. 5. This curve was prepared by plotting the slope of the standards obtained in Figure 4 against their mol wt. The mol wt of the 11.3S spore storage protein (*arrow*) was determined from this standard plot.

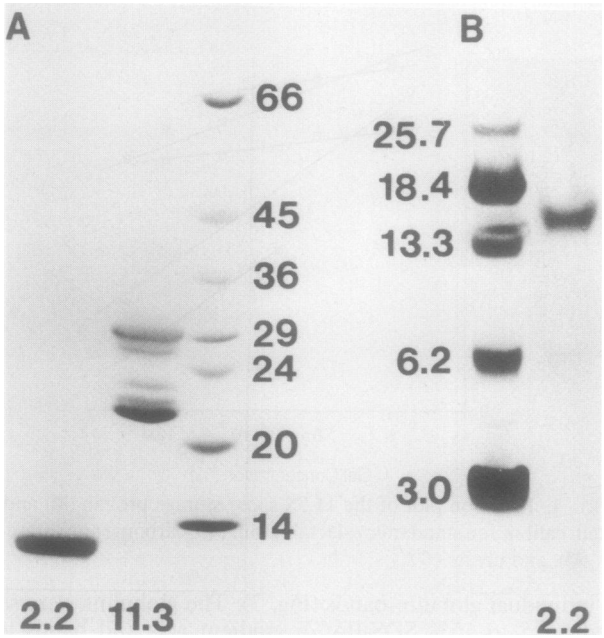

FIG. 6. (A) SDS-PAGE profiles of the 2.2S and 11.3S proteins on a 5 to 15% gradient gel. The gel was stained with CBB and the mol wt of protein standards (M_r) is indicated by numbers on the right. (B) SDS-PAGE reducing profile of the 2.2S protein separated on a 15% gel. Fifteen mg of protein were loaded per lane and the gel was silver-stained. The mol wt of protein standards (M_r) is indicated by the numbers on the left.

amino acid composition of the two proteins is presented in Table I. An average mol wt of 32,000 for the 2.2S and 232,000 for the 11.3S protein was used to calculate the mM/g protein for each amino acid. The 2.2S fraction registered high levels of aspartic and glutamic acids, with these two residues representing 26.1% of the total composition. The large amount of ammonia (22.5%) detected suggests that the majority of these residues are in the form of asparagine and glutamine, which have neutral polar groups. Since the 2.2S protein has few acidic residues that are

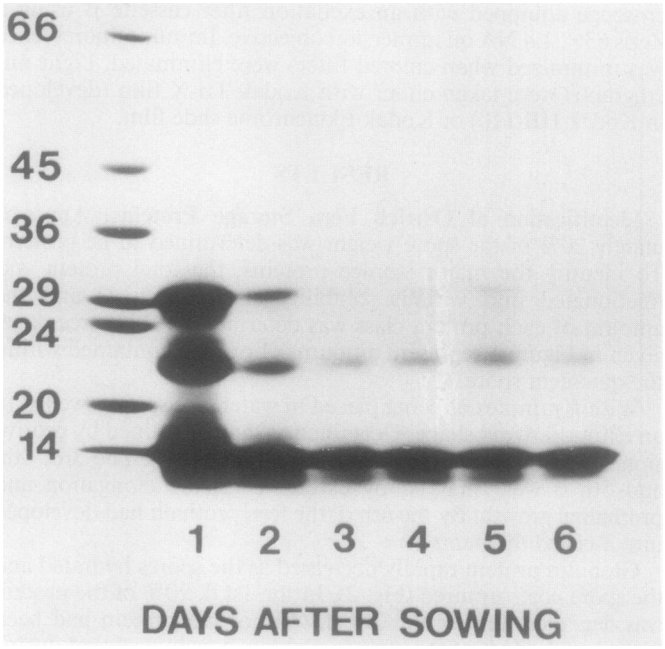

FIG. 7. SDS-PAGE profiles of spore globulins on a 5 to 15% gradient gel. Globulins were extracted from an equivalent amount of spores (200 mg initial dry weight) at various times after sowing. The gel was stained with CBB and the mol wt of protein standards (M_r) is indicated by numbers on the left.

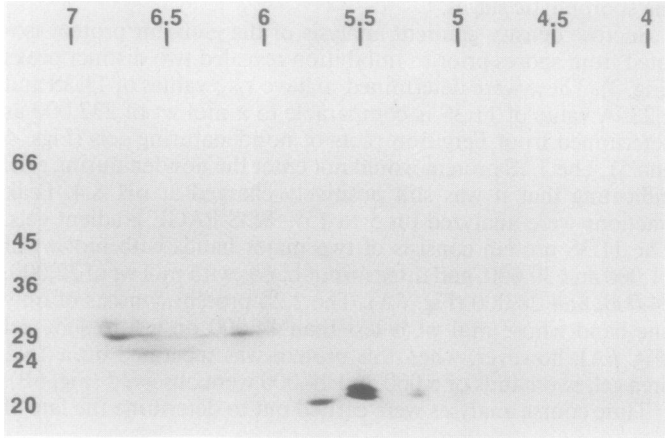

FIG. 8. Two-dimensional gel electrophoresis of ostrich fern 11.3S protein. A 15-mg aliquot of protein was loaded on the IEF gel. Sucrose gradient fraction number 20 as shown in Figure 3 was used for this experiment. Gel was silver-stained as described. Mol wt are indicated on the left and the pH gradient is shown along the top.

not present as amides, the amount of arginine present (10%) makes this protein very basic. This was confirmed by IEF analysis. The 11.3S fraction was also rich in aspartic and glutamic acids, representing 19.5% of the total composition. However, the observed levels of basic amino acids (10.4%) and ammonia (2.2%) are consistent with the relatively neutral pI range observed for the 11.3S protein.

Immunolocalization of the 2.2S and 11.3S Proteins within the Spore. When sections of quiescent fern spores were reacted with anti-spore globulin, followed by the fluorescent secondary antibody, fluorescence was localized in discrete organelles (Fig. 9, C and D) that were judged to be protein bodies on the basis of their morphological changes during spore germination (6). These organelles, which are 2 to 5 μm in diameter, are positioned just

Table I. *Amino Acid Composition of the 2.2S and 11.3S Storage Proteins*

Amino Acid	Protein	
	2.2S	11.3S
	mmol/g protein	
Lysine	0.017	0.067
Arginine	0.579	0.337
Histidine	0.102	0.098
Ammonia	1.331	0.107
Aspartic acid	0.436	0.354
Glutamic acid	1.109	0.577
Glycine	0.592	0.349
Serine	0.662	0.219
Threonine	0.070	0.093
½-Cysteine	ND[a]	ND
Tyrosine	0.051	0.045
Alanine	0.349	0.258
Valine	0.082	0.162
Leucine	0.238	0.228
Isoleucine	0.067	0.104
Proline	0.124	0.130
Phenylalanine	0.025	0.126
Methionine	0.076	0.059
Tryptophan	nd	ND

[a] Not determined.

inside a thin layer of small (>1 μm) lipid droplets that line the inner spore wall, the intine. Larger plastids, up to 15 μm in diameter, are centrally located within the spore. No fluorescence was detected when preimmune serum was used as the primary antibody. Similar fluorescent patterns were observed when anti-2.2S or anti-11.3S serum was used as the primary antibody. While autofluorescence of lipid droplets, plastids, and the intine was observed (Fig. 9B), this fluorescence was red at an excitation wavelength of 460 nm compared to the yellow-green color of FITC-labeled material.

DISCUSSION

It is of interest from both a chemical and phylogenetic viewpoint to find that the major storage proteins in ostrich fern spores are globulins. These salt-soluble proteins are a significant reserve in the seeds of many flowering plants, including most legumes and crucifers (14).

The 11.3S protein is similar in several respects to the legumin-like protein contained in seeds of a variety of dicotyledons and monocotyledons (7). Its mol wt, subunit composition, solubility, and relatively neutral pI are similar to those of legumin. The extent of this similarity is evident from two-dimensional gel analysis of the 11.3S protein, which reveals the degree of charge heterogeneity of the various subunits. The ratio of amino acids in the 11.3S spore storage protein is consistent with the range of values observed among the various 11S seed globulin proteins.

The 2.2S fern spore globulin is also similar to some seed globulins. Smaller seed globulins, 2 to 4S, are a more diverse group than the 11S legumin-like proteins. The majority of these smaller globulins can be classified into two distinct subgroups based upon pI differences. In most legumes that contain a small globulin, the 2 to 4S protein has a slightly acidic pI (7), whereas in some crucifers the small globulin (1.7S) has a very basic pI, usually above pH 10. The 2.2S globulin of *Matteuccia* exhibits many of the characteristics of the 1.7S cruciferous proteins of *Brassica* (5) and *Raphanus* (19, 20). In addition to a basic pI, the 2.2S *Matteuccia* protein is similar to the crucifer proteins in mol wt, subunit composition, and solubility profile. There are substantial differences in the amino acid composition when the fern 2.2S and the 1.7S protein of *Brassica* are compared, but there is evidence that substantial compositional heterogeneity exists even among various species of *Brassica* (11). The trivial names "Pteridophytin" and "Matteuccian" have been assigned to designate the 11.3S and 2.2S protein from spores of *Matteuccia*.

The degradation profile of the spore proteins is consistent with enzymic data on proteolytic activity occurring during imbibition and germination of ostrich fern spores (3). Peak endopeptidase, aminopeptidase, and carboxylase activities were detected 12 to 24 h after spore imbibition, the time corresponding to the initiation of globulin degradation. Correlations between enzymic activity and storage protein degradation have been documented in a variety of diverse angiosperm seeds (21). The amino acids released during spore storage protein breakdown are most likely used as a nitrogen-rich source of materials for future synthesis, a role similar to that of seed storage proteins.

In recent years, the metabolic events associated with germinating fern spores have received considerable attention. Throughout imbibition and germination, fern spores have been shown to be active in DNA synthesis, transcription, translation, protein degradation, lipid catabolism, and starch accumulation (6, 9, 10, 16, 22, 24). Many of these processes have been associated with responses to light, temperature, and other environmental factors (24). In addition, phytate is present in ostrich fern spores and metabolized during germination (6). Phytate, or phytic acid, is commonly found in seed protein bodies as crystalloids and provides a reserve of phosphate that is used during germination. These earlier metabolic studies and the results reported here indicate that fern spores share many of the biochemical characteristics of seeds.

The similarity between ostrich fern spore storage proteins and angiosperm seed storage proteins is closer than one might have expected, considering the distant relationships between ferns and flowering plants. Storage proteins from *Bacillus*, *Anabena*, and *Macrozamia* have been reported that fulfill the same developmental role as storage proteins in the angiosperm seeds, but they show little chemical homology to seed proteins of flowering plants.

Three storage proteins have been isolated and extensively characterized from the bacterial spores of *Bacillus megaterium* (26). These storage polymers comprise 15% of the total spore protein. They are soluble in dilute acid and their accumulation is regulated at the transcriptional level. All three proteins have low mol wt (7–10,000), basic pI (>9.8), alanine as the NH_2-terminal amino acid, lack cysteine, cystine, and tryptophan, and appear to be encoded by a multigene family (12). A critical difference between these bacterial storage proteins and seed storage proteins is that these bacterial proteins are also capable of binding to nucleic acids (26).

In *Anabena cylindrica*, storage protein is sequestered in the cells of this prokaryote in cyanophycin granules (27). Large numbers of these granules accumulate not only in spores but also in stationary phase cultures. The protein contains only two amino acids, aspartic acid and arginine, which are present in a 1:1 molar ratio. This polymer contains two polypeptides of 25,000 and 100,000 whose amounts can vary (28). The characteristics of this storage compound clearly differentiate it from storage proteins found in seed-bearing plants.

One of the few gymnosperm storage proteins whose composition and structural properties have been determined is from seeds of the cycad, *Macrozamia communis* (1). This cycad, which has been included in the prephanerogams, a relict group of ancient gymnosperms, was found to have a unique 10.9S globulin protein termed macrozin. Macrozin consists of three 44 Mr polypeptides that are disulfide-bonded to form a 126,000 subunit; two subunits are jointed together to form the 260,000 (10.9S) oligomer. While

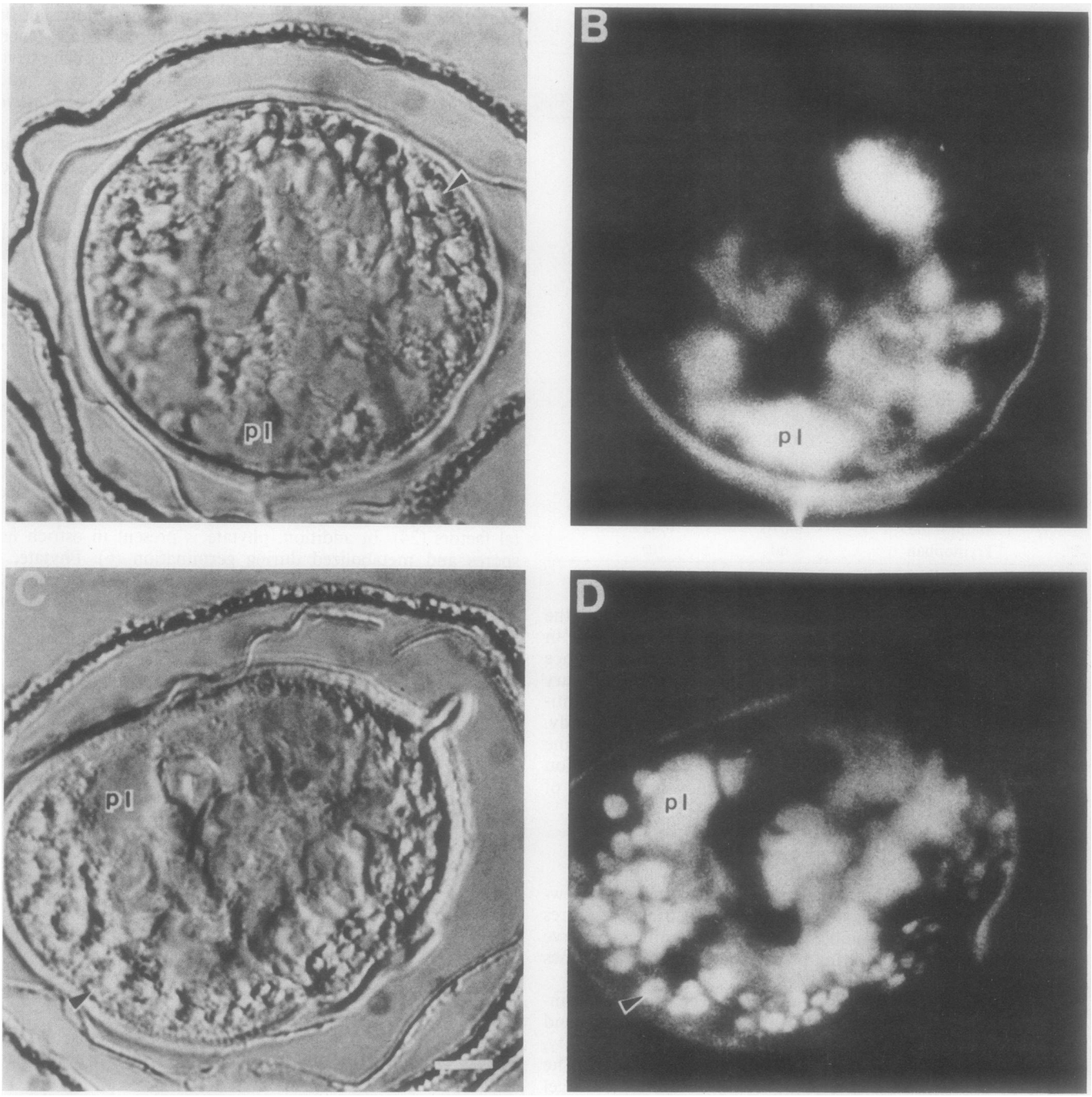

FIG. 9. Corresponding differential interference contrast (A and C) and fluorescence (B and D) light micrographs of quiescent ostrich fern spores prepared for indirect immunofluorescence. The spores were treated with either pre-immune serum (A and B) or anti-spore globulin serum (C and D) as the primary antibody. All spores were treated with FITC-labeled anti-rabbit IgG. Plastids (pl) exhibit autofluorescence (red) under this treatment. FITC fluorescence (green) is limited to protein bodies (*arrows*). Bar = 10 μm.

this protein is similar to other known globulin proteins in its isoelectric point range (6 to 7.5) and amino acid composition, structurally it is quite different from globulins identified in other gymnosperms (18) or angiosperms (14).

The occurrence of these diverse types of storage proteins suggests that wide latitude exists in their structure and composition. Nevertheless, these proteins all function adequately as storage materials. On the other hand, there is considerable evidence that selective evolutionary pressures have acted to reduce structural variation (8). The situation is not clear and more study of storage proteins from diverse plant groups is required in order to assess how these proteins have changed during evolution.

Ferns could be especially important for this work. They consist of a diverse group of vascular plants considered to have evolved from ancient trimerophytes that also gave rise to the seed plants. While there is no direct evidence for a common evolutionary origin for storage proteins, the results presented here provide a basis for continuing investigations to determine the degree of homology between spore and seed proteins.

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