JOURNAL OF BACTERIOLOGY, Aug. 1985, p. 696–703
0021-9193/85/080696-08$02.00/0

Vol. 163, No. 2

Synthesis and Localization of a Development-Specific Protein in Sclerotia of *Sclerotinia sclerotiorum*†

GERALDINE M. RUSSO AND JAMES L. VAN ETTEN*

Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68583-0722

Received 4 February 1985/Accepted 23 April 1985

A development-specific protein (SSP) makes up about 35 to 40% of the total protein in sclerotia of the fungus *Sclerotinia sclerotiorum*. The protein consists of three charge isomers, with one isomer making up 80 to 90% of the total. In vitro translation of poly(A)$^+$ RNA isolated from cells in early stages of sclerotia formation revealed that 44% of the amino acids incorporated was into SSP. In vivo- and in vitro-synthesized forms of SSP migrated at identical rates on both isoelectric focusing and denaturing polyacrylamide gels, indicating that SSP was not synthesized as a larger precursor. This was significant because SSP accumulated in membrane-bound, organellelike structures which resemble protein bodies found in seeds of many higher plants.

The fungus *Sclerotinia sclerotiorum*, which overwinters as macroscopic resting structures termed sclerotia, is pathogenic to more than 380 plant species (35). While overwintering, the sclerotia undergo a physiological conditioning process (12, 43) that leads to functional maturity (38). In the spring, they germinate by forming apothecia which then release large numbers of ascospores (termed carpogenic germination); the ascospores serve as the primary source of inoculum for infecting plants (e.g., see references 1 and 13). *S. sclerotiorum* can also germinate by an alternate mechanism which does not require physiological conditioning. In this case the sclerotia germinate by forming vegetative hyphae and, ultimately, more sclerotia (termed myceliogenic germination).

We have discovered that a development-specific protein (designated SSP) makes up 35 to 40% of the total sclerotial protein (37). This protein, which has an apparent molecular size of 36 kilodaltons, is not present in vegetative cells of the fungus but accumulates rapidly during sclerotium formation.

This report shows that (i) SSP exists as a series of charge isomers, a major isomer and two minor isomers with isoelectric points of 6.0, 5.8, and 6.2, respectively, (ii) SSP synthesis is probably under transcriptional control, (iii) SSP mRNA accounts for at least 44% of the translatable mRNA in presclerotia, (iv) SSP is not synthesized as a larger precursor, and (v) SSP accumulates in membrane-bound organellelike structures.

MATERIALS AND METHODS

Materials. [^{35}S]methionine and ^{14}C-labeled protein molecular weight standards were purchased from Amersham Corp. and Bethesda Research Laboratories, Inc., respectively. Goat anti-rabbit immunoglobulin G (IgG) labeled with colloidal gold was purchased from SPI Supplies. Normal goat serum was purchased from Calbiochem-Behring.

Fungus strain and culture conditions. The source of the *S. sclerotiorum* isolate and the growth conditions were described previously (37). Morphological stages associated with sclerotium formation include (i) the appearance of white tufts of thick mycelial growth (sclerotia primordia), (ii) the enlargement and hardening of these mycelial tufts and the appearance of an exudate on the surface of developing sclerotia (early presclerotia), (iii) the continued hardening of the presclerotia owing to rind formation and the beginning of melanin pigmentation (late presclerotia), and (iv) the drying of the exudate and completion of rind formation and pigmentation (mature sclerotia). It requires 24 to 48 h for cultures to proceed from stage i to stage iii.

Purification of SSP. Dried mature sclerotia were ground in a Wiley mill, and the powder was extracted with 0.1 M acetic acid at 25°C for 24 h. The acetic acid extract, which contained SSP, was collected by low-speed centrifugation; we removed large polysaccharides by centrifuging the extract in a Beckman SW41 rotor at 38,000 rpm, 5°C, for 6 h. The supernatant was dialyzed against 50 mM Tris chloride (pH 7.2), and SSP was precipitated with 60 to 80% $(NH_4)_2SO_4$. The precipitate was suspended in 50 mM Tris chloride (pH 7.2), dialyzed against the same buffer, and chromatographed on a Bio-Gel P-100 (100/200-mesh; Bio-Rad Laboratories) column. SSP fractions were pooled, dialyzed against 10 mM Tris chloride (pH 8.0), and chromatographed on a Cellex-D (Bio-Rad) anion-exchange cellulose column equilibrated in the same buffer. Protein was eluted with a 0 to 0.2 M KCl gradient in 10 mM Tris chloride (pH 8.0). SSP, which eluted at approximately 0.1 M KCl, was dialyzed against 10 mM Tris chloride (pH 7.4) and lyophilized.

Isolation of polysomes and poly(A)$^+$ RNA. Cells (250 to 300 mg [wet weight]) were mixed with 5 ml of breakage buffer (0.05 M Tris chloride, 0.06 M KCl, 0.03 M $MgCl_2$ [pH 7.8]) and disrupted in a Brinkmann Polytron for 15 to 20 s at full speed. After low-speed centrifugation to remove cell debris, a polysomal pellet was obtained by layering the supernatant over 1 ml of a 540-mg/ml concentration of sucrose in the same buffer and centrifuging in a Beckman SW50.1 rotor for 4 h at 45,000 rpm and 4°C.

The polysomes were suspended in 0.5 ml of breakage buffer and centrifuged on 12.5 to 50.0% (wt/vol) linear sucrose density gradients equilibrated with breakage buffer in a Beckman SW41 rotor for 75 min at 38,000 rpm and 4°C. Gradients were scanned photometrically at A_{254}; ribosome fractions were collected and pelleted by centrifugation in an SW41 rotor for 5 h at 38,000 rpm and 4°C. Each ribosomal pellet was suspended in 100 μl of buffer (5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid], 10

* Corresponding author.

† Published with the approval of the director as paper no. 7685, Journal Series, Nebraska Agricultural Experiment Station.

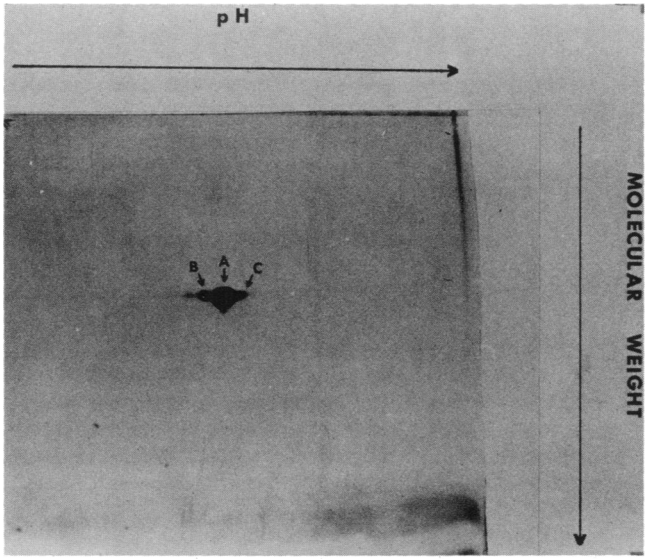

FIG. 1. Two-dimensional gel electrophoresis of 50 μg of purified SSP. Electrophoresis in the first dimension was from pH 4 to 7. Electrophoresis in the second dimension was on a linear 8-to-20% denaturing polyacrylamide gel. Arrow A indicates the major SSP charge isomer; arrows B and C indicate minor SSP charge isomers. Proteins were silver stained after electrophoresis.

mM potassium acetate, 3 mM magnesium acetate [pH. 7.6]) and translated in a reticulocyte in vitro translation system.

Poly(A)$^+$ RNA was isolated from early presclerotia and vegetative cell polysomes by one to three passes over an oligo(dT) cellulose column by the procedure of Larkins et al. (20). Potassium acetate was added to the final RNA eluant to a concentration of 0.2 M. RNA was precipitated with ethanol at −20°C and finally suspended in water.

In vitro translation. Polysomes or poly(A)$^+$ RNA (1.0 to 1.5 μg) isolated from polysomes was translated in a rabbit reticulocyte in vitro system by the procedure of Paterson et al. (31) as modified by Schleif and Wensink (40). The reticulocyte lysate (Green Hectares) was first digested with micrococcal nuclease (Worthington Diagnostics) (32, 40). The final concentrations of K^+ and Mg^{2+} in a 25-μl translation reaction volume were 112 and 1.2 mM, respectively. RNA was heated for 3 min at 65°C and quickly cooled on ice immediately before it was added to the translation mixture.

Translation assays were incubated for 1 h at 33°C, and the reactions were stopped by placing the tubes on ice. Each sample was centrifuged in a Beckman Airfuge at 131 kPa (19 lb/in^2) of air pressure for 13 min to pellet polysomes with nascent polypeptides. The amount of [^{35}S]methionine incorporated into protein in the supernatant was determined by trichloroacetic acid precipitation and processed as described by Bollum (5). Separate samples were either used directly in immunoprecipitation experiments, diluted with an equal volume of 2× electrophoresis denaturing sample buffer and analyzed on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels, or treated with 10 volumes of acetone to precipitate proteins for isoelectric focusing.

Serological experiments. Antiserum against purified SSP was raised in randomly bred rabbits. At 7-day intervals, the rabbits were injected three times intramuscularly in the calf or thigh and one time subcutaneously with 1 to 2 mg of SSP plus an equal concentration of methylated bovine serum albumin. The antigen mixture for intramuscular injections was suspended in Freund complete adjuvant. The rabbits were exsanguinated 3 weeks after the last injection. The anti-SSP IgG fractions were isolated (30), taken up in 10 mM KPO_4–15 mM NaCl (pH 7.5), and lyophilized.

Immunoprecipitation assays were as follows. Formalin-fixed *Staphylococcus* A cells (BRL immunoprecipitin) were suspended (10% [wt/vol]) in 10 mM Tris chloride–150 mM NaCl–1% (wt/vol) Triton X-100 (pH 7.2). To decrease nonspecific binding, each radioactively labeled protein antigen was pretreated with 10% (wt/vol) *Staphylococcus* cells in a ratio of 6 parts antigen:1 part *Staphylococcus* cells (vol/vol) for 20 min at room temperature. The samples were then centrifuged in a Beckman Airfuge at 145 kPa (21 lb/in^2) of air pressure for 9 min. The supernatant (antigen) was collected, and the radioactivity was measured. Serum or IgG preparations and ovalbumin carrier protein solutions were also centrifuged in a Beckman Airfuge at 131 kPa (19 lb/in^2) of air pressure for 1 h to pellet aggregated protein.

The assay mixture, containing buffer (10 mM Tris chloride–150 mM NaCl [pH 7.2]), detergents (1% [wt/vol] Triton X-100–1% [wt/vol] sodium deoxycholate), 60 μg of ovalbumin as carrier protein, antigen, and antibody in a total volume of 200 μl, was incubated at room temperature for 2 h and then overnight at 4°C. A total of 100 μl of 10% (wt/vol) *Staphylococcus* A cells was then added to each sample and

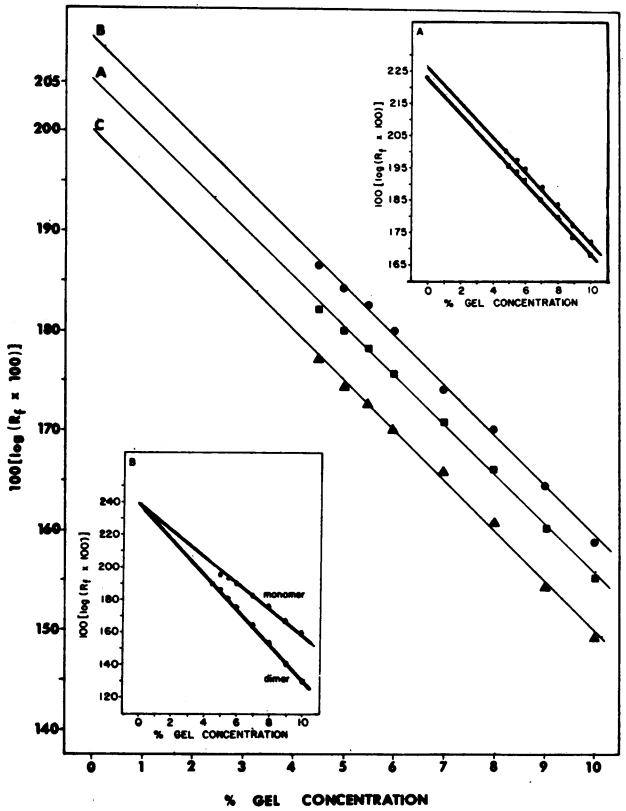

FIG. 2. Characterization of SSP as a family of charge isomers. All proteins were electrophoresed on a series of nondenaturing polyacrylamide gels. Chicken egg albumin (insert A) and bovine serum albumin (insert B) were electrophoresed and analyzed as representative charge and molecular weight isomers, respectively. The three SSP charge isomers are lettered analogously to the protein bands in Fig. 1.

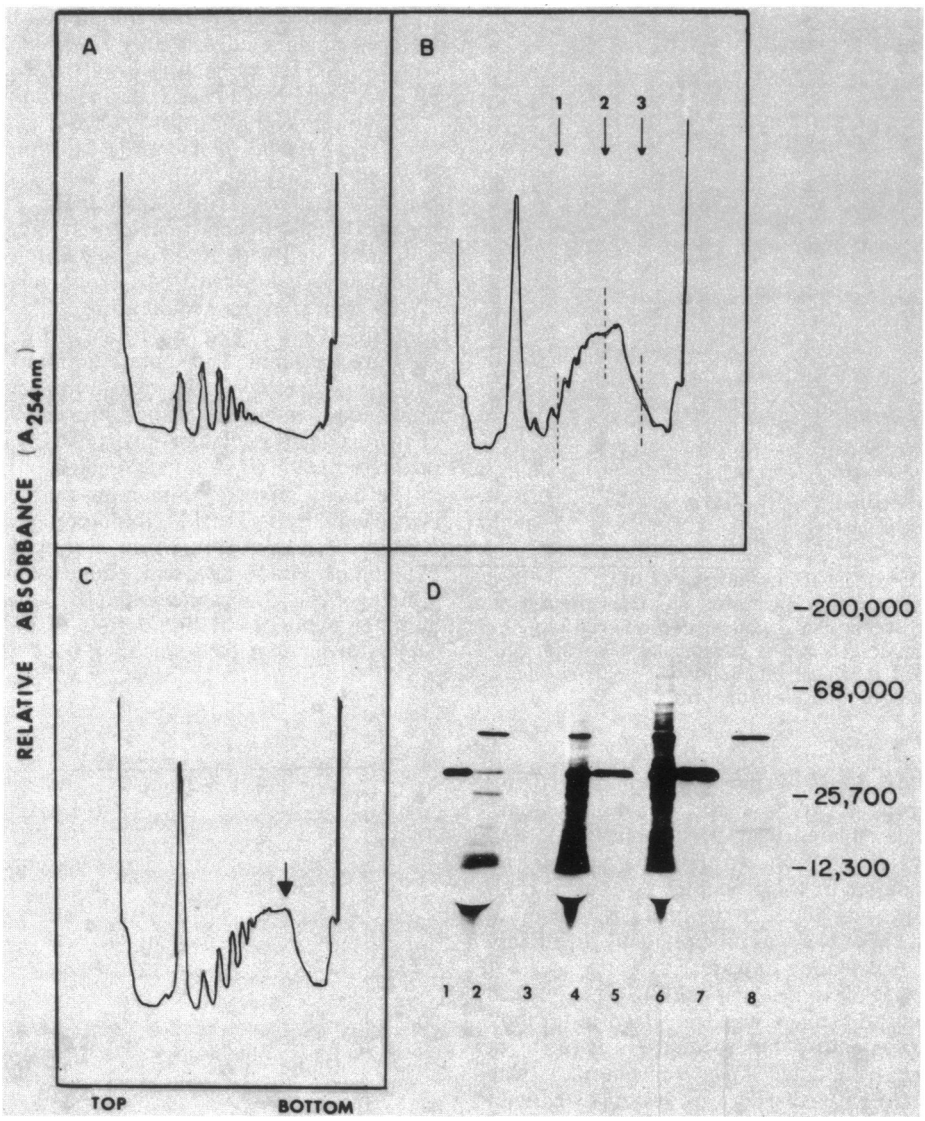

FIG. 3. Sucrose density gradient analysis of polyribosomes isolated from *S. sclerotiorum* (A) vegetative cells, (B) early presclerotia, and (C) late presclerotia. (D) Fluorogram of a linear 8-to-20% SDS-polyacrylamide gel. Polysomes from early presclerotia were fractionated into three parts as indicated in panel B, pelleted, resuspended, and translated in vitro in a rabbit reticulocyte system. Lane 1, ^{3}H-labeled in vivo-synthesized SSP. Lane 2, ^{35}S-labeled in vitro translation products translated from polysome fraction 1, 66,700 cpm layered. Lane 3, same protein as in lane 2 but immunoprecipitated with anti-SSP IgG, 19,300 cpm layered. Lane 4, ^{35}S-labeled translation products from polysome fraction 2, 66,700 cpm layered. Lane 5, proteins from lane 4 immunoprecipitated with anti-SSP IgG, 25,300 cpm layered. Lane 6, ^{35}S-labeled translation products from polysome fraction 3, 66,700 cpm layered. Lane 7, proteins from lane 6 immunoprecipitated with anti-SSP IgG, 37,900 cpm layered. Lane 8, ^{35}S-labeled translation products from endogenous mRNA present in the rabbit reticulocyte lysate. Note that the counts per minute layered on the gel in lanes 3, 5, and 7 represents the counts per minute immunoprecipitated from 66,700 cpm of the appropriate translation sample. Molecular weight standards are indicated to the right of the figure.

incubated at room temperature for 1 h. The immunoprecipitate was collected by centrifugation in a Beckman Microfuge B for 5 min at 4°C and washed four times with 10 mM Tris chloride–150 mM NaCl–1% (wt/vol) Triton X-100 (pH 7.2). Each precipitate was taken up in 70 to 75 μl of electrophoresis sample denaturing buffer (see below), boiled for 5 min, and centrifuged in a Beckman Microfuge B for 5 min at room temperature. The supernatant was collected from the dissociated antigen-antibody complexes, and the immunoprecipitated radioactive protein was measured.

Electrophoresis of proteins. One- and two-dimensional gel electrophoresis was performed by the method of Laemmli (18) and O'Farrell (28), respectively. Isoelectric focusing tube gels contained 2% (wt/vol) Ampholine carrier ampholytes (LKB Instruments, Inc.) (1% pH 5 to 7 and 1% pH 3.5 to 10). Protein samples for one-dimensional gel analysis were denatured in 0.028 M Tris chloride (pH 6.8)–6.5% (wt/vol) glycerol–10% (wt/vol) sucrose–4.3% (wt/vol) SDS–0.36 M 2-mercaptoethanol–0.34 M dithiothreitol–0.67 mM EDTA–0.35% (wt/vol) bromophenol blue by boiling for 3 min and immediately layered onto gels. Protein samples for isoelectric focusing were taken up in 9.5 M urea–2% (wt/vol) Nonidet P-40–2% Ampholine carrier ampholytes (1% pH 5 to 7 and 1% pH 3.5 to 10)–5% (wt/vol) 2-mercaptoethanol. After electrophoresis, the gels were stained with either Coomassie blue or silver (27). In some

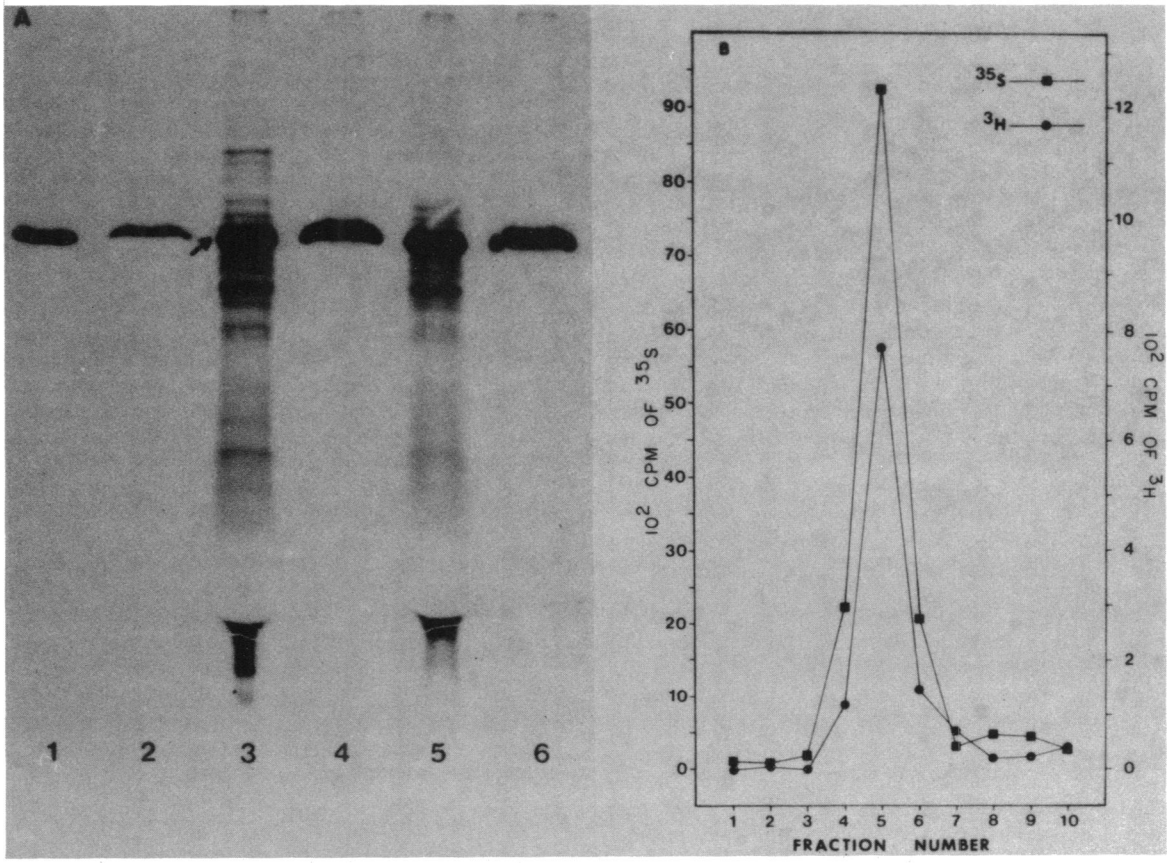

FIG. 4. (A) Analysis of ^{3}H-labeled in vivo-synthesized SSP and ^{35}S-labeled in vitro-synthesized SSP on a one-dimensional 15% SDS-polyacrylamide gel. Lane 1, ^{3}H-labeled purified SSP. Lane 2, same protein as in lane 1 but immunoprecipitated with anti-SSP IgG. Lane 3, in vitro ^{35}S-labeled translation products from presclerotial polysomal poly(A)$^+$ RNA. The arrow indicates the position of the SSP. Lane 4, same protein as in lane 3 but immunoprecipitated with anti-SSP IgG. Lane 5, a mixture of ^{3}H-labeled purified SSP and ^{35}S-labeled presclerotial polysomal poly(A)$^+$ RNA translation products. Lane 6, same proteins as in lane 5 but immunoprecipitated with anti-SSP IgG. (B) A 2-cm region encompassing the SSP band in lane 6 of panel A was excised, sliced into 2-mm sections, solubilized, and counted for ^{3}H and ^{35}S.

cases the gels were sliced into 2-mm sections, solubilized in NCS tissue solubilizer (Amersham/Searle), and counted.

Charge isomers of SSP were characterized by electrophoresing the nondenatured protein on a series of one-dimensional polyacrylamide slab gels (4.5, 5.0, 5.5, 6.0, 7.0, 8.0, 9.0, or 10.0% polyacrylamide) as described in Sigma Chemical Co. Technical Bulletin no. MKR-137. Nondenatured protein standards (Sigma) were taken up in 50 mM NaCl–1 mM $NaPO_4$ (pH 7.0), and purified SSP was taken up in 10 mM Tris chloride (pH 7.4). After electrophoresis, the dye front on each gel was marked with India ink, and the gels were stained and destained with the same solutions as for denaturing gels. However, after destaining, the gels were equilibrated in 7% acetic acid for several hours before the measurement of protein and dye front migration distances for calculation of the Ferguson plots (15) was performed.

Transmission electron microscopy and immunohistochemistry. *S. sclerotiorum* mycelium, early presclerotia, and late presclerotia were minced with a razor blade into approximately 2-mm^3 sections and fixed in 2% (vol/vol) glutaraldehyde in 0.1 M $NaPO_4$ (pH 6.8) for 2 h at 4°C. The specimens were rinsed briefly in phosphate buffer and postfixed in 1% (wt/vol) osmium tetroxide in 0.1 M $NaPO_4$ (pH 6.8) for 1 h at 4°C. The postfixed material was washed several times before being dehydrated in a graded ethanol series and embedded in Epon 812 (Electron Microscopy Science). Thin sections (ca. 120 nm) were cut on an LKB Ultramicrotome III with a diamond knife.

Sections for immunocytochemistry were etched by immersion in alcoholic NaOH for 2 min followed by jet-stream rinsing with absolute ethanol (14). The tissue sections were air dried and then floated on a saturated aqueous solution of sodium metaperiodate for 2 h at room temperature (4). The sections were rinsed by floating them on six changes of distilled water and air dried.

The two-step staining procedure of Lin and Langenberg (22) was used with several modifications. The etched, sodium metaperiodate-treated, Epon-embedded tissue sections were treated at 20°C as follows. They were (i) incubated in 3.3% (vol/vol) normal goat serum in 10 mM $NaPO_4$–15 mM NaCl–20 mM NaN_3 (pH 7.5) (phosphate-buffered saline [PBS]-azide) for 20 min; (ii) incubated in rabbit anti-SSP IgG diluted 1:200 in PBS-azide for 20 min; (iii) washed six times with 1% (vol/vol) normal goat serum in PBS-azide, 3 min each wash; (iv) incubated in gold-labeled goat anti-rabbit IgG complex diluted 1:15 in 20 mM Tris chloride–150 mM NaCl–20 mM NaN_3 (pH 8.2) containing 1% (wt/vol) ovalbumin for 20 min; (v) washed six times with 1% (vol/vol) normal goat serum in PBS-azide, 3 min each wash; (vi) washed twice with PBS-azide, 3 min each wash; and (vii)

washed twice with distilled water, 3 min each wash. The diluted gold-labeled goat anti-rabbit IgG complex was centrifuged at low speed (250 × *g*) to remove aggregates before use in step iv.

All thin sections were stained with saturated aqueous uranyl acetate and lead citrate (36) and viewed in a Philips 201 transmission electron microscope at 60 kV.

Other procedures. Protein concentrations were determined by the method of Lowry et al. (25) with bovine serum albumin as a standard. RNA concentrations were determined by assuming that 1 mg of RNA per ml had an A_{260} of 24. Purified proteins were labeled in vitro with [^{3}H]potassium borohydride by the method of Kumarasamy and Symons (17). Gels containing radioactive proteins were prepared for fluorography by the method of Bonner and Laskey (6) and placed in light-tight presses with Kodak X-Omat XAR-5 film; the film was exposed for various times at −80°C.

RESULTS

Characterization of SSP. Two-dimensional gel electrophoresis of purified SSP revealed three protein bands with the same apparent molecular size of 36 kilodaltons but with different isoelectric points (Fig. 1). A major band (labeled A in Fig. 1) made up about 80 to 90% of the total and had a pI of 6.0; two minor bands (B and C in Fig. 1) had pIs of 5.8 and 6.2, respectively. To determine whether the two minor bands were charge isomers of SSP or unrelated proteins, purified SSP was electrophoresed on a series of nondenaturing gels, and the relative mobility (R_f) of each protein was measured as a function of the acrylamide concentration (Fig. 2). Because the three bands in the SSP profile gave parallel Ferguson plots, as did chicken egg albumin charge isomers (insert A in Fig. 2), we concluded that the two minor bands were probably charge isomers of SSP.

Polysomes. Polysomes were isolated from vegetative cells, early presclerotia, and late presclerotia and analyzed on sucrose density gradients (Fig. 3A to C). Interestingly, there was a prominent peak in the 10-to-11-ribosome region of the polysome profiles from early and late presclerotia (indicated by the arrow in Fig. 3C); this peak was absent in polysomes from vegetative cells. Because rapid SSP synthesis begins during early stages of sclerotium formation (37), it seemed likely that this 10-to-11-polyribosome peak would contain large amounts of SSP mRNA. To test this possibility, we separated polysomes from early presclerotia into three fractions and translated them in vitro; the total translation products as well as products precipitated with anti-SSP IgG were electrophoresed on linear 8-to-20% denaturing polyacrylamide gels. As seen in Fig. 3D, the fraction containing the 10-to-11-polyribosome fraction was enriched in SSP mRNA.

In vitro translation of SSP mRNA. The amount of SSP mRNA in early presclerotia was estimated by translating isolated poly(A)$^+$ RNA from the total polysomal fraction. The translation products were either precipitated with trichloroacetic acid to estimate total translatable mRNA or precipitated with anti-SSP IgG followed by resuspension and trichloroacetic acid precipitation to estimate SSP mRNA. The average of three separate experiments indicated that 44% (range, 39 to 49%) of the total [^{35}S]methionine incorporated into the protein was precipitated by anti-SSP IgG. The value may actually be higher because immunoprecipitation conditions were not optimized. When the same experiment was conducted with poly(A)$^+$ RNA isolated from vegetative polysomes, none of the in vitro-synthesized translation products was precipitated with anti-SSP IgG (data not shown). The anti-SSP IgG is specific for SSP, because electrophoresis of the anti-SSP IgG precipitate on polyacrylamide gels gave only one band (Fig. 3D, lanes 5 and 7), which migrated at the same rate as purified in vivo-synthesized SSP (Fig. 3D, lane 1). We conclude that SSP mRNA makes up at least 44% of the total polysomal poly(A)$^+$ RNA present in presclerotia and that translatable SSP mRNA is either absent or present at very low levels in vegetative cells.

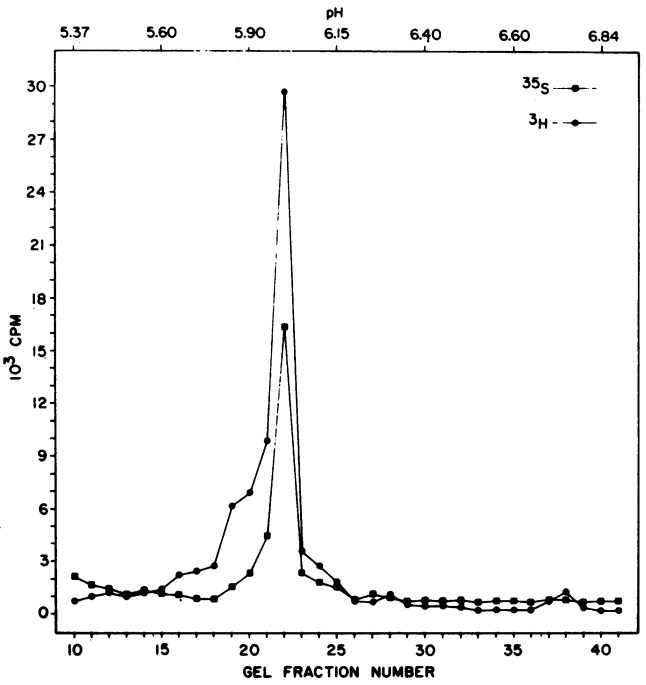

FIG. 5. Isoelectric focusing of a mixture of ^{3}H-labeled in vivo-synthesized SSP and ^{35}S-labeled in vitro-synthesized SSP. After focusing, the gel was sliced into 2-mm sections, solubilized, and counted for ^{3}H and ^{35}S.

As reported below, SSP accumulates in organellelike protein bodies in the sclerotia, and so we anticipated that SSP would initially be synthesized as a precursor protein, possibly containing a signal peptide. Therefore, we attempted to separate in vitro-synthesized SSP from purified SSP. First, in vitro-synthesized SSP migrated at the same rate as purified SSP when analyzed on a linear 8-to-20% denaturing polyacrylamide gel (Fig. 3D). Second, in vitro-synthesized SSP labeled with [^{35}S]methionine was electrophoresed either separately or together with purified ^{3}H-labeled SSP on 12%, 15%, and 18% SDS-polyacrylamide gels. In all three gels, the ^{3}H-labeled in vivo-synthesized SSP migrated to exactly the same position as the ^{35}S-labeled in vitro-synthesized SSP. For convenience, only the 15% gel is shown in Fig. 4A. After each experiment a 2-cm region containing the anti-SSP IgG-precipitated SSP band was excised from the lane containing the mixture of in vitro- and in vivo-synthesized SSP and sliced into 2-mm sections. These sections were solubilized and counted for ^{3}H and ^{35}S. As shown in Fig. 4B for the 15% gel, in vitro- and in vivo-synthesized SSP migrated at the same rates. Identical results were obtained with the 12 and 18% gels. In separate experiments with proteins of known molecular weights we believe that we would have detected differences in migration

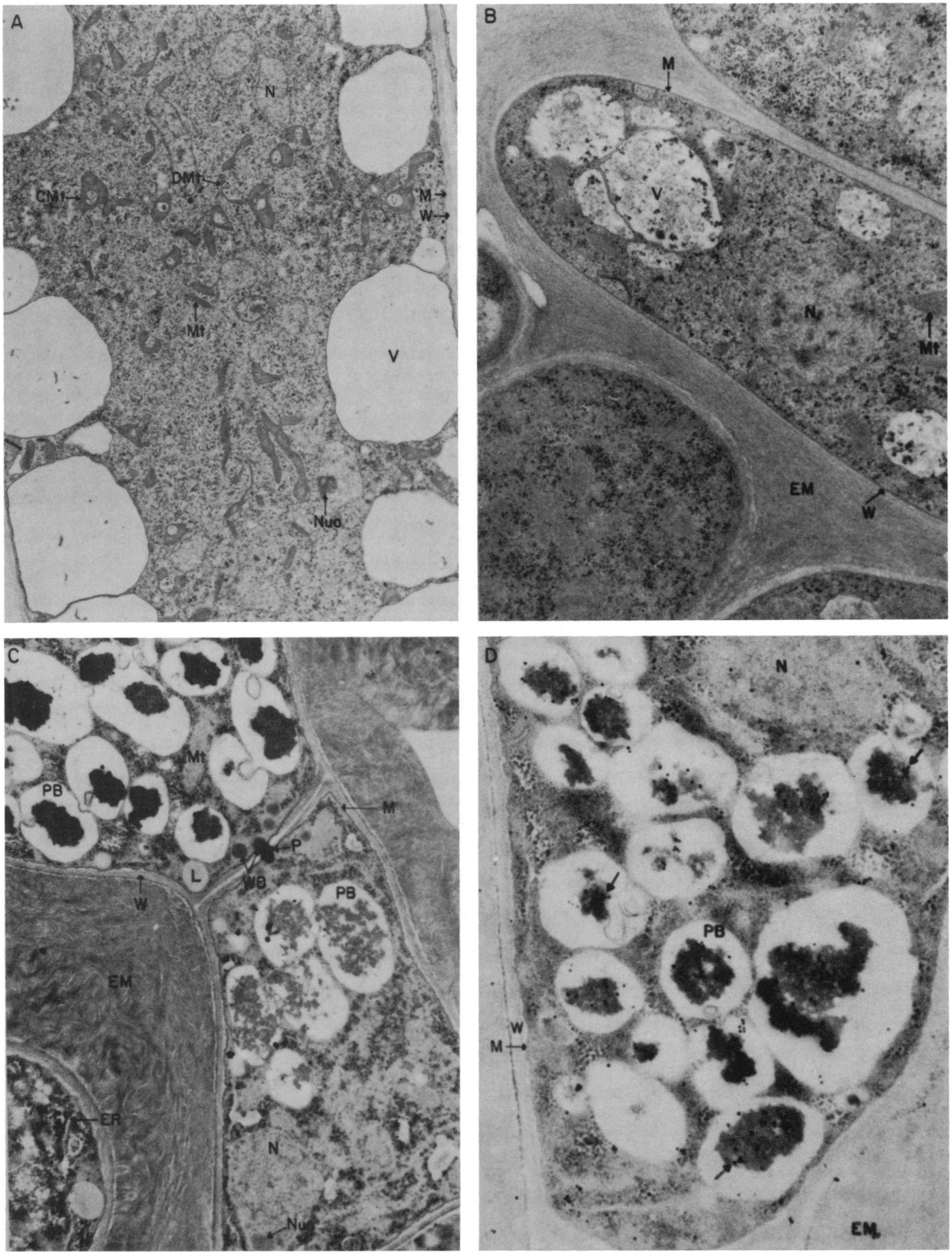

FIG. 6. Morphology of (A) vegetative cells (×8,300), (B) early presclerotia (×20,000), and (C) late presclerotia (×18,400) and (D) localization of SSP within the sclerotium of *S. sclerotiorum*. In panel D thin sections of late presclerotia (×28,400) were reacted with rabbit anti-SSP IgG as the primary antibody and gold-labeled goat anti-rabbit IgG as the secondary antibody. The gold particles (indicated by the arrows in panel D) were localized to the protein bodies (PB). Abbreviations: N, nucleus; Nuo, nucleolus; Mt, mitochondria; CMt, concentric mitochondria; DMt, dumbbell-shaped mitochondria; ER, endoplasmic reticulum; L, lipid vesicle; P, pore; WB, Worin bodies; V, vacuole; W, cell wall; M, cell membrane; EM, extracellular matrix.

if the in vitro-synthesized SSP was five amino acids longer than the final product.

Finally, ^{3}H-labeled SSP and ^{35}S-labeled in vitro-synthesized SSP were coelectrophoresed on isoelectric focusing gels. After focusing, the gels were sliced into 2-mm sections, solubilized, and counted. The amounts of ^{3}H and ^{35}S peaked in fraction 22, corresponding to a pH of 6.0, which is the isoelectric point of the major charge isomer of SSP (Fig. 5). Thus, SSP is apparently not synthesized as a precursor that is posttranslationally modified by amino acid cleavage or by phosphorylation, glycosylation, ribosylation, methylation, etc.

Localization of SSP. Figures 6A to C show transmission electron micrographs of vegetative cells, early presclerotia, and late presclerotia, respectively. Of particular interest was the presence of globous vacuoles in all of the cells. In early presclerotial cells (Fig. 6B), many of these vacuoles contained a loose network of aggregated, moderately electron-dense material. This material increased in volume and became more electron dense and tightly aggregated in the vacuoles of late presclerotial cells (Fig. 6C). The vacuoles of vegetative cells (Fig. 6A) did not contain any electron-dense material and were larger than those of early and late presclerotia.

Thin sections of late presclerotia were treated with rabbit anti-SSP IgG as the primary antibody and gold-labeled goat anti-rabbit IgG as the secondary antibody. As shown in Fig. 6D, the gold was localized to the vacuoles containing the electron-dense material. In contrast, gold staining of vegetative cells was random and equivalent to background, i.e., with nonimmune rabbit IgG as the primary antibody. In addition, all of the controls suggested by Bendayan (3) were conducted, and the results support the conclusion that the anti-SSP IgG specifically reacted with the electron-dense material present in the vacuoles of presclerotia. We conclude that SSP accumulates in these membrane-bound organelles and that these organelles are protein bodies.

DISCUSSION

We reported previously that SSP accumulates rapidly during sclerotium formation in *S. sclerotiorum* and ultimately makes up 35 to 40% of the total protein present in mature sclerotia (37). In this paper we established that SSP mRNA makes up ca. 44% of the total translatable poly(A)$^{+}$ RNA in developing sclerotia. Because translatable SSP mRNA and SSP protein were not detected in vegetative cells, SSP is development specific and probably under transcriptional control.

SSP exists as three charge isomers; a major isomer makes up 80 to 90% and two minor isomers each make up 5 to 10% of the total. Molecular weight or charge heterogeneity or both are characteristic of a number of development-specific storage proteins present in the seeds of many higher plants (8, 16, 19, 21). The charge heterogeneity of many of these seed storage proteins is due at least in part, if not totally, to multigene families encoding similar but not identical polypeptides. We do not know whether the charge isomers of SSP are encoded by a family of genes.

SSP accumulates in organelles which morphologically resemble protein bodies in the seeds of higher plants (2, 24). Consequently, we anticipated that SSP, like many storage proteins in seeds, would be synthesized as a precursor protein, i.e., have a signal sequence, which is cleaved to yield mature SSP during transport into these protein bodies. However, attempts to detect such a precursor failed. Cleavable signal sequences are typically 15 to 30 amino acids in length (41). The methods we used should have detected a protein that is five amino acids longer than SSP. Thus, we conclude that if an SSP precursor exists it is not much larger than the final product.

It is more likely that SSP is a member of the rare group of exported proteins which lack a cleavable signal sequence. Other examples in eucaryotic organisms include ovalbumin, a major protein found in eggs of fowl (29), and opsin, the apoprotein of the visual pigment rhodopsin present in vertebrate rod photoreceptor cells (39). There is evidence that ovalbumin contains an internal signal sequence; however, its exact location in the primary sequence of the protein is still controversial (7, 23, 26).

Although protein bodies are commonly found in the seeds of higher plants, there are only a few reports of such organelles in fungi. From ultrastructural and histochemical studies, Bullock et al. (9–11) identified protein bodies in sclerotia of *Sclerotinia minor*, a fungus which is closely related to *S. sclerotiorum*. In this report, we established the existence of protein bodies in *S. sclerotiorum* sclerotia which accumulate SSP. We also have evidence that the development-specific protein muiridin, which makes up 25% of the spore protein in *Botryodiplodia theobromae* (42), is localized in protein bodies (manuscript in preparation). Muiridin is actively degraded during spore germination and thus presumably functions as a storage protein, i.e., serves as a source of amino acids during spore germination (33).

Although its function is not known, SSP may serve as a source of nitrogen or amino acids or both during apothecia formation. Two observations are consistent with this hypothesis. First, SSP decreases significantly during carpogenic germination but not during myceliogenic germination (37). Second, from ultrastructural and histochemical evidence Bullock et al. (11) suggest that the protein in the protein bodies of *S. minor* sclerotia is degraded during carpogenic germination. We previously identified a major development-specific sclerotial protein in *S. minor* which is similar, but not identical, to SSP (34). In all probability, this development-specific protein accumulates in the sclerotial protein bodies of *S. minor* and is degraded during carpogenic germination.

ACKNOWLEDGMENTS

We thank Ellen Ball for help in producing SSP antibody and Kit Lee for assistance with the electron microscopy.

This investigation was supported in part by Public Health Service grant AI-08057 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Abawi, G. S., and R. G. Grogan.** 1975. Source of primary inoculum and effects of temperature and moisture on infection of beans by *Whetzelinia sclerotiorum*. Phytopathology **65**:300–309.
2. **Ashton, F. M.** 1976. Mobilization of storage proteins of seeds. Annu. Rev. Plant Physiol. **27**:95–117.
3. **Bendayan, M.** 1984. Protein A-gold electron microscopic immunocytochemistry: methods, applications, and limitations. J. Electron Microsc. **1**:243–270.
4. **Bendayan, M., and M. Zollinger.** 1983. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J. Histochem. Cytochem. **31**:101–109.
5. **Bollum, F. J.** 1967. Filter paper disk techniques for assaying radioactive macromolecules. Methods Enzymol. **12B**:169–173.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.

7. **Brell, W. A., and H. F. Lodish.** 1982. Ovalbumin utilizes an NH_2-terminal signal sequence. J. Biol. Chem. **257**:4578–4582.
8. **Brown, J. W. S., D. R. Ersland, and T. C. Hall.** 1982. Molecular aspects of storage protein synthesis during seed development, p. 3–42. *In* A. A. Khan (ed.), The physiology and biochemistry of seed development, dormancy and germination. Elsevier Biomedical Press, Amsterdam.
9. **Bullock, S., A. E. Ashford, and H. J. Willetts.** 1980. The structure and histochemistry of sclerotia of *Sclerotinia minor* Jagger. II. Histochemistry of extracellular substances and cytoplasmic reserves. Protoplasma **104**:333–351.
10. **Bullock, S., H. J. Willetts, and A. E. Ashford.** 1980. The structure and histochemistry of sclerotia of *Sclerotinia minor* Jagger. I. Light and electron microscope studies on sclerotial development. Protoplasma **104**:315–332.
11. **Bullock, S., H. J. Willetts, and A. E. Ashford.** 1983. The structure and histochemistry of sclerotia of *Sclerotinia minor* Jagger. III. Changes in ultrastructure and loss of reserve materials during carpogenic germination. Protoplasma **117**:214–225.
12. **Coley-Smith, J. R., and R. C. Cooke.** 1971. Survival and germination of fungal sclerotia. Annu. Rev. Phytopathol. **9**:65–92.
13. **Cooke, G. E., J. R. Steadman, and M. G. Boosalis.** 1975. Survival of *Whetzelinia sclerotiorum* and initial infection of dry edible beans in western Nebraska. Phytopathology **65**:250–255.
14. **Erlandsen, S. L., J. A. Parsons, and C. B. Rodning.** 1979. Technical parameters of immunostaining of osmicated tissue in epoxy sections. J. Histochem. Cytochem. **27**:1286–1289.
15. **Hedrick, J. L., and A. J. Smith.** 1968. Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**:155–164.
16. **Higgins, T. J. V.** 1984. Synthesis and regulation of major proteins in seeds. Annu. Rev. Plant Physiol. **35**:191–221.
17. **Kumarasamy, R., and R. H. Symons.** 1979. The tritium labeling of small amounts of protein for analysis by electrophoresis on sodium dodecyl sulfate-polyacrylamide slab gels. Anal. Biochem. **95**:359–363.
18. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
19. **Larkins, B. A.** 1981. Seed storage proteins: characterization and biosynthesis, p. 449–489. *In* P. K. Stumpf and E. E. Conn (ed.), The biochemistry of plants: a comprehensive treatise, vol. 6. Academic Press, Inc., New York.
20. **Larkins, B. A., R. A. Jones, and C. Y. Tsai.** 1976. Isolation and *in vitro* translation of zein messenger ribonucleic acid. Biochemistry **15**:5506–5522.
21. **Leaver, C. J. (ed.).** 1980. Genome organization and expression in plants, p. 203–289. Plenum Publishing Corp., New York.
22. **Lin, N.-S., and W. G. Langenberg.** 1983. Immunohistochemical localization of barley stripe mosaic virions in infected wheat cells. J. Ultrastruct. Res. **84**:16–23.
23. **Lingappa, V. R., J. R. Lingappa, and G. Blobel.** 1979. Chicken ovalbumin contains an internal signal sequence. Nature (London) **281**:117–121.
24. **Lott, J. N. A.** 1980. Protein bodies, p. 589–623. *In* P. K. Stumpf and E. E. Conn (ed.), The biochemistry of plants: a comprehensive treatise, vol. 1. Academic Press, Inc., New York.
25. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
26. **Meek, R. L., K. A. Walsh, and R. D. Palmiter.** 1982. The signal sequence of ovalbumin is located near the NH_2 terminus. J. Biol. Chem. **257**:12245–12251.
27. **Morrissey, J. H.** 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. **117**:307–310.
28. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
29. **Palmiter, R. D., J. Gagnon, and K. A. Walsh.** 1978. Ovalbumin: a secreted protein without a transient hydrophobic leader sequence. Proc. Natl. Acad. Sci. U.S.A. **75**:94–98.
30. **Palmiter, R. D., T. Oka, and R. T. Schimke.** 1971. Modulation of ovalbumin synthesis by estradiol-17B and actinomycin D as studied in explants of chick oviduct in culture. J. Biol. Chem. **246**:724–737.
31. **Paterson, B. M., D. J. Marciani, and T. S. Papas.** 1977. Cell-free synthesis of the precursor polypeptide for avian myeloblastosis virus DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **74**:4951–4954.
32. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient RNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
33. **Petersen, G. R., K. R. Dahlberg, and J. L. Van Etten.** 1983. Biosynthesis and degradation of storage protein in spores of the fungus *Botryodiplodia theobromae*. J. Bacteriol. **155**:601–606.
34. **Petersen, G. R., G. M. Russo, and J. L. Van Etten.** 1982. Identification of major proteins in sclerotia of *Sclerotinia minor* and *Sclerotinia trifoliorum*. Exper. Mycol. **6**:268–273.
35. **Purdy, L. H.** 1979. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution, and impact. Phytopathology **69**:875–880.
36. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
37. **Russo, G. M., K. R. Dahlberg, and J. L. Van Etten.** 1982. Identification of a development-specific protein in sclerotia of *Sclerotinia sclerotiorum*. Exp. Mycol. **6**:259–267.
38. **Saito, I.** 1977. Studies on the maturation and germination of sclerotia of *Sclerotinia sclerotiorum* (LIB) DE BARY, a causal fungus of bean stem rot. Rep. Hokkaido Prefect. Agric. Exp. Stn. **26**:1–106.
39. **Schechter, I., Y. Burstein, R. Zemell, E. Ziv, F. Kantor, and D. S. Papermaster.** 1979. Messenger RNA of opsin from bovine retina: isolation and partial sequence of the *in vitro* translation product. Proc. Natl. Acad. Sci. U.S.A. **76**:2654–2658.
40. **Schleif, R. F., and P. C. Wensink.** 1981. Practical methods in molecular biology. Springer-Verlag, New York.
41. **Silhavy, T. J., S. A. Benson, and S. D. Emr.** 1983. Mechanisms of protein localization. Microbiol. Rev. **47**:313–344.
42. **Van Etten, J. L., S. N. Freer, and B. K. McCune.** 1979. Presence of a major (storage?) protein in dormant spores of the fungus *Botryodiplodia theobromae*. J. Bacteriol. **138**:650–652.
43. **Willetts, H. J., and J. A.-L. Wong.** 1980. The biology of *Sclerotinia sclerotiorum*, *S. trifoliorum*, and *S. minor* with emphasis on specific nomenclature. Bot. Rev. **46**:101–165.