

Purification of Maize Pollen Exines and Analysis of Associated Proteins¹

Christopher H. Chay, Erich G. Buehler, Judith M. Thorn, Thomas M. Whelan, and Patricia A. Bedinger*

Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599–3280

ABSTRACT

Zea mays (maize) pollen exines have been purified with the use of differential centrifugation and sucrose gradients, followed by mild detergent and high salt treatment. The final exine fraction is highly purified from other organelles and subcellular structures as assayed by transmission electron microscopy. Using mature maize pollen as the starting material, 0.2 to 0.3% of the total pollen protein remained associated with the exine fraction throughout the purification. Seven abundant sodium dodecyl sulfate-extractable proteins are detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the final fraction. Amino acid analysis reveals that one of the proteins contains a substantial amount of hydroxyproline, a characteristic of some primary cell wall proteins. The amino acid composition of the 25-kD protein strongly implies that it is an arabinogalactan protein. When exines are purified from earlier pollen developmental stages, a subset of the proteins found in the mature pollen exine is seen.

The extracellular matrix that makes up the primary plant cell wall is largely composed of polysaccharides and other polymers, including cellulose, hemicellulose, pectic compounds, and lignin. In addition to polysaccharides, there are tightly bound proteins that presumably serve a structural purpose in the cell wall, including the hydroxyproline-rich extensins and AGPs² (4), glycine-rich proteins (5), and cysteine-rich proteins (3). The exact function of each of these proteins within the cell wall is not known, but is an area of active research.

It is only recently that maize (*Zea mays*) cell wall proteins have been studied. The graminaceous monocots do not have a high level of hydroxyproline in their cell walls. However, a hydroxyproline-rich protein has been purified recently from maize suspension culture cells (15) and from maize pericarp (13). In addition, the gene for a proline or hydroxyproline-rich cell wall protein has been cloned from maize (29). It appears that the purified threonine-rich maize cell wall protein may be related to the protein encoded by the cDNA clone (16).

The angiosperm pollen wall is a highly specialized extra-

cellular matrix; its functions include the protection of the male gametophyte after dehiscence into the environment, as well as facilitation of the interaction of the pollen grain with the pistil prior to fertilization (12). A highly resistant substance called sporopollenin is the major component of the outer pollen wall. Although sporopollenin has been studied for over 150 years (14), the chemical structure of this polymer has been elusive (10, 22).

The synthesis of the exine, or outer pollen wall, begins at a very early point in pollen development. There is some evidence that exine patterning elements are inserted into the membrane of the diploid meiocyte just prior to meiosis (25). After meiosis, while the tetrad of haploid microspores is still enclosed in a callose wall, the microspores elaborate an extracellular structure called the primexine (11) or glycocalyx (23) that determines the pattern of pollen wall formation. The pore or aperture region is also defined at the tetrad stage, distinguishable at the TEM level by a gap in the primexine.

After release of the tetrad of microspores from the callose enclosure, sporopollenin is rapidly deposited on the microspore surface. It is thought that tapetal cells, which are filled with ER, golgi, and vesicles at this stage, secrete sporopollenin precursors into the anther locule (20); these precursors are then polymerized onto the glycocalyx template to form the exine. The typical ektexine, or outer exine, is a trilamellar structure (12). The outermost layer is called the tectum, which is often decorated with spinules. The interior layer is called the foot layer. Rod-like baculae or collumelae form connections between the tectum and foot layer. Late in development, a cellulosic/pectic layer called the intine is formed next to the pollen plasma membrane.

Although well described at the cytological level (7, 26), very little is known about the molecular or biochemical aspects of pollen wall formation and structure. Given the elaborate structural features of the angiosperm exine and the precedence of primary cell wall proteins, it is likely that there are structural protein components of the pollen wall in addition to sporopollenin. In this article, we report the extensive purification of maize pollen exines and the characterization of several tightly associated, SDS-extractable proteins.

MATERIALS AND METHODS

Materials

Zea mays inbred line Ky21 was grown in a glass greenhouse with supplemental lighting. Chemicals were purchased from Sigma Chemical Co.

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² Abbreviations: AGP, arabinogalactan protein; TEM, transmission electron microscopy.

Exine Preparation

Starting material was either developmentally staged microspores (2) or mature pollen. All steps were performed at 4°C. Briefly, the microspores or pollen were disrupted using a French press at 10,000 p.s.i. in 0.1 M Tris, pH 8, containing protease inhibitors leupeptin, pepstatin, and chymostatin at a final concentration of 5 µg/mL and PMSF at 2.4 mM. The broken cells were centrifuged at 10,000g to produce a crude wall fraction (pellet) and a cytoplasmic fraction (supernatant). The crude walls were washed in 0.1 M Tris-Cl, pH 8, several times and then loaded onto a continuous 20 to 60% (w/v) sucrose gradient. The pollen walls banded at about 50% sucrose after 30 min at 30,000 rpm in an SW41 rotor. The wall band was collected, washed, and treated with 0.05% deoxycholate and 0.1 M Tris-Cl, pH 8, for 2 h. After washing in 0.1 M Tris-Cl, pH 8, the walls were then treated with 2 M NaCl and 0.1 M Tris-Cl, pH 8, overnight. The final wall fraction was obtained after washing in 0.1 M Tris-Cl, pH 8. Proteins were extracted by boiling samples in SDS sample buffer (0.0625 M Tris, pH 6.8, 10% w/v glycerol, 5% v/v E1-mercaptoethanol, and 2.3% w/v SDS), and protein concentrations of extracts were determined using the method of Schaffner and Weissmann (24).

TEM

Samples were fixed in 63% ethanol, 5% glacial acetic acid, 5% formaldehyde overnight. After fixation, samples were washed several times in cacodylate buffer and treated with 2% osmium tetroxide for 1.5 h. Samples were then dehydrated in a graded ethanol series and placed in several changes of acetone. A warm epon/araldite mixture consisting of 14 mL of dodecylsuccinic anhydride, 6.2 mL of epon, 8.1 mL of araldite 506 epoxy resin, and 0.75 mL of dibutyl phthalate was made. After cooling, 32 drops of tri(dimethyl amino methyl)phenol were added and mixed. Samples were then embedded in this mixture. Sections were made using a Sorvall MT-2 ultramicrotome with a diamond knife. Sections were stained with uranyl acetate followed by lead citrate. Sections were observed using a Zeiss EM10-CA microscope.

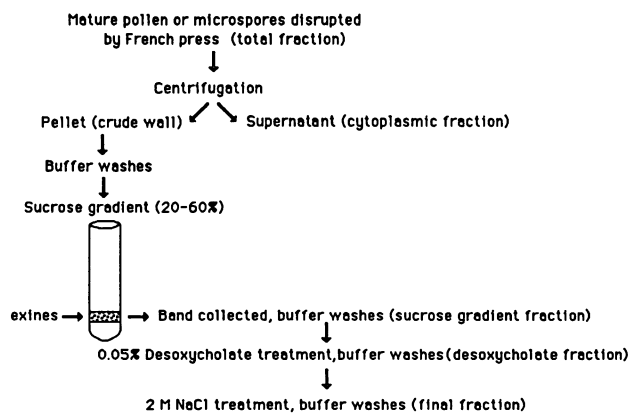


Figure 1. Flow diagram of exine purification.

Table I. Protein in Exine Purification Fractions

Starting material is mature pollen.			
Fraction	Protein ^a	Percent ^a	Average Percent ^b
	mg		
Total	1146.6	100	100
Cytoplasmic	1105.4	96.4	86.7
Crude wall	67	5.85	5.71
Sucrose gradient	3.74	0.37	0.47
Deoxycholate wash	3.25	0.28	0.4
NaCl wash (final)	2.16	0.189	0.28

^a From a single typical preparation. ^b From the average of four preparations.

Gel Electrophoresis

Pelleted pollen walls were resuspended in 1X SDS sample buffer and heated at 100°C for 3 min. After centrifugation in a microcentrifuge for 5 min, the supernatant containing solubilized proteins was drawn off and transferred to a fresh tube. Samples were analyzed by SDS-PAGE on 10 to 18% (w/v) polyacrylamide gradient gels. Gels were stained with either Coomassie blue or silver nitrate (19).

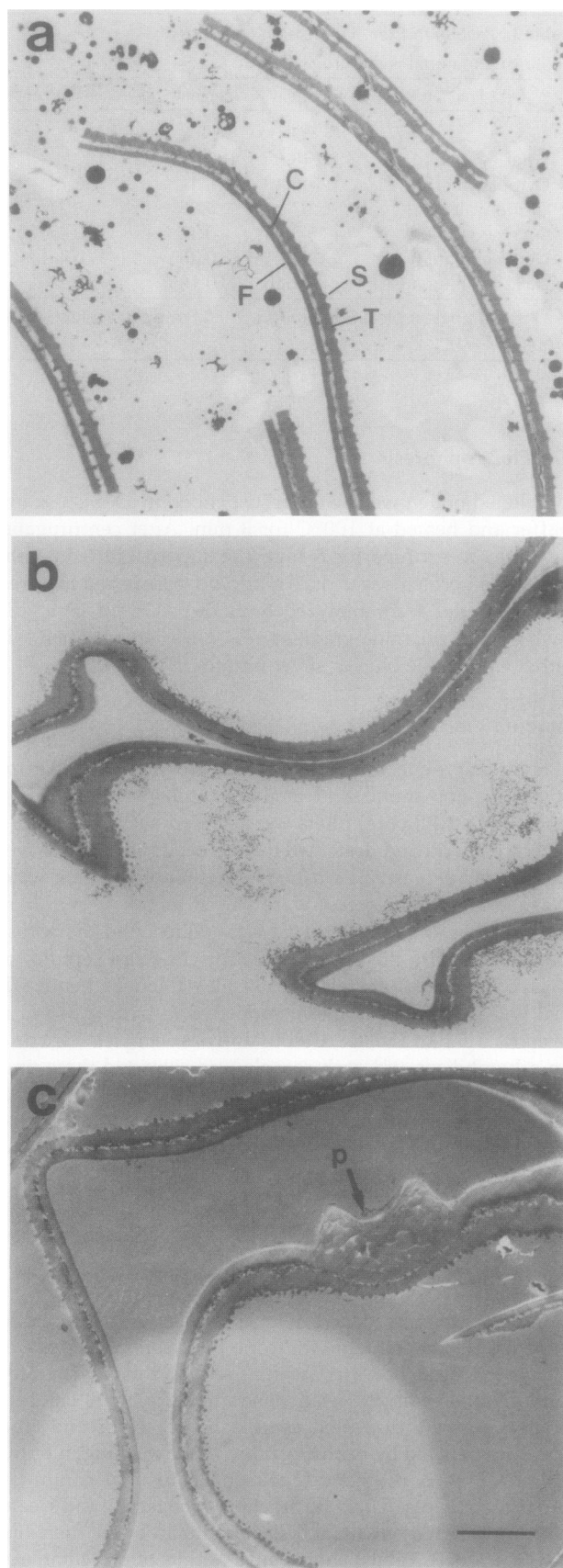
Amino Acid Analysis

Hydroxyproline was assayed using the method of Drozd et al. (6). For amino acid analysis, proteins transferred to Immobilon (Millipore) filters were stained with Ponceau S in 10% acetic acid and destained in 10% acetic acid. Bands were excised and extensively washed in methanol and then water. Washed membrane pieces were placed in 6 × 50 mm borosilicate tubes, 10 µL of 6 N HCl were added, and the borosilicate tubes were placed in a 25-mL screw cap septum vial (Pierce Chemical) containing 500 µL of liquid phenol. The vial was flushed with argon, capped, and hydrolyzed in an oven for 1.5 h at 150°C. After hydrolysis, samples were dried on a lyophilizer and each sample was extracted three times with 200 µL of 0.1 N HCl in 30% methanol. The extracts were combined, dried in a vacuum centrifuge, and derivatized with phenylisothiocyanate for analysis on a Waters Associates Pico Tag amino acid analyzer.

RESULTS

Purification of Pollen Walls from *Z. mays*

Developmentally staged microspores (2) or mature pollen were the starting material for exine purification. Exines were purified as described in "Materials and Methods." The preparation scheme is depicted diagrammatically in Figure 1. Briefly, cells are disrupted using a French press and crude walls are pelleted by centrifugation. After washing in buffer, the crude walls are centrifuged on 20 to 60% continuous sucrose gradients. The wall fraction is collected and treated with mild detergent (0.05% desoxycholate) and then high salt (2 M NaCl). Table I shows the amounts of protein in each step of the purification. Sucrose gradient banding of exines is a very efficient fractionation step. The exine-associated



proteins in our final fraction represent about 0.2 to 0.3% of the total protein in the mature pollen starting material.

The purity of the exine preparations was examined using TEM. The crude wall fraction has many organelles, starch granules, and membranous structures in addition to the pollen walls (Fig. 2a). It should be noted that the intine layer does not remain associated with the exine in this preparation. The exines after sucrose gradient centrifugation are greatly purified, but still have some loosely associated material (Fig. 2b). The final exine fraction, after desoxycholate and salt treatment, has no visible contaminants (Fig. 2c).

The protein components of the pollen wall fraction were analyzed using SDS-PAGE as described in "Materials and Methods." As shown in Figure 3, the sucrose gradient appears to make a major contribution to exine purification. There are seven major proteins in the final exine fraction. These proteins are apparently 85, 80, 60, 32, 25, 17, and 15 kD in size. The 25-kD protein stains faintly with Coomassie brilliant blue, but darkly with silver (Ag lane).

Deglycosylation of pollen walls and extracts of pollen walls according to the method of Mani et al. (18) revealed a change in migration of most pollen wall proteins on SDS-PAGE, indicating that at least some of the pollen wall proteins are glycosylated (data not shown). Only the 17- and 15-kD proteins were not affected by the deglycosylation treatment.

It has been shown that a soluble form of extensin can be eluted from primary cell walls of some plants using divalent cations such as calcium. To examine whether calcium-elutable protein(s) were detectable in pollen exines, sucrose gradient-purified exine fractions were treated with calcium (0.2 M CaCl_2 , 0.1 M Tris-Cl, pH 7.5), compared with sodium (0.4 M NaCl, 0.1 M Tris-Cl, pH 7.5) and buffer only (0.1 M Tris-Cl, pH 7.5). As the results shown in Figure 4 indicate, we did not observe any calcium-specific elution of proteins from exines. It appears that the 15- and 17-kD proteins are elutable with either salt or buffer treatment, and may therefore be less tightly associated with the exines than, for example, the 25-kD protein.

Amino Acid Analysis of the 25-kD Protein

Pollen walls were treated with acid and analyzed for hydroxyproline as described by Drozd et al. (6). A low but consistent amount of hydroxyproline was detectable. Amino acid analysis of the 25-kD protein using HPLC, as described in "Materials and Methods," is shown in Table II. This analysis indicates that the 25-kD protein (which stains faintly with Coomassie blue, but stains strongly with silver) is rich in hydroxyproline residues (11.95%). When added to the proline fraction (7.31%), the total proline is over 19%. The 25-kD protein is also fairly serine- and glycine-rich (10.9% and 15.27%, respectively).

Figure 2. TEM analysis of exine fractions. a, Crude pollen walls (pellet after French press and centrifugation at 10,000g). S, Spinules; T, tectum; C, collumellae; F, foot layer. b, Pollen walls after sucrose gradient centrifugation. c, Pollen walls after both desoxycholate and NaCl treatments. p indicates pore. Bar is 5 μm .

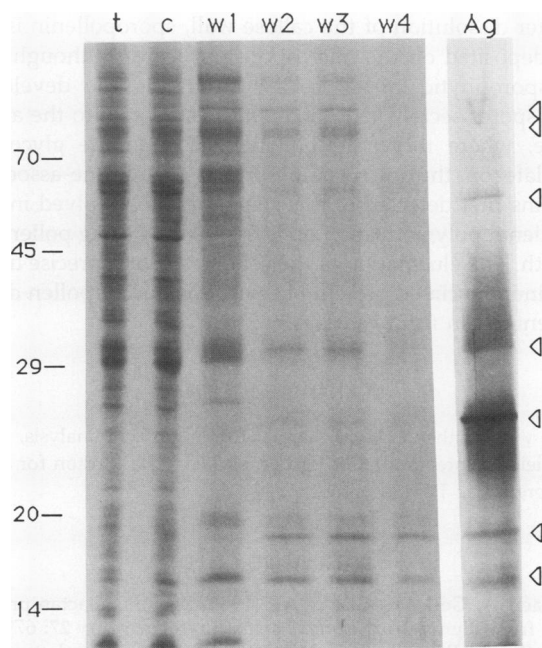


Figure 3. SDS-PAGE of fractions in exine preparation. t, Total pollen protein (100 μ g of protein); c, cytoplasmic fraction (100 μ g); w1, crude wall fraction (50 μ g); w2, sucrose gradient fraction (50 μ g); w3, desoxycholate fraction (50 μ g); w4, final wall fraction (50 μ g).

Table II. Amino Acid Composition of the 25-kD Exine Binding Protein^a

Amino Acid	Composition mol %
Ala	7.71
Arg	4.40
Asx	5.01
Cys	2.18
Glx	10.66
Gly	15.27
His	0.97
Hyp	11.95
Ile	1.70
Leu	3.84
Lys	3.55
Met	2.06
Phe	1.21
Pro	7.31
Ser	10.9
Thr	5.61
Trp	ND ^b
Tyr	2.14
Val	3.51

^a The average of amino acid analysis from four different mature pollen exine preparations. ^b Not detectable.

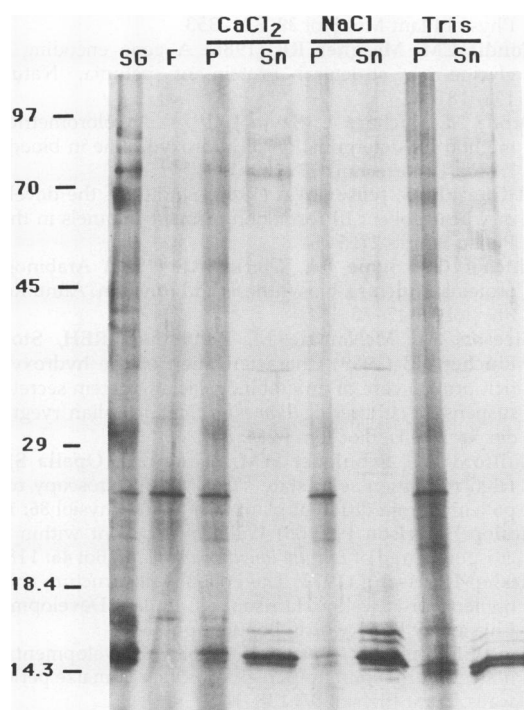


Figure 4. Elution of proteins by salt and buffer treatment. Sucrose gradient-purified exines were treated overnight at 4°C in 0.1 M Tris-Cl, pH 7.5, with either 0.2 M CaCl₂, 0.4 M NaCl, or buffer only. After pelleting exines by centrifugation, supernatants (400 μ L out of 4 mL) were precipitated with cold 10% TCA prior to electrophoresis. SG, Sucrose gradient fraction; F, final fraction; P, pellet; and Sn, supernatant.

Exine Proteins from Several Developmental Stages

Microspores and pollen were developmentally staged, as described by Bedinger and Edgerton (2). Exines were purified from young microspores (soon after meiosis), microspores with a single large vacuole (just prior to microspore mitosis), starch-filled young pollen, and mature pollen. It can be seen in Figure 5 that 32-, 25-, and possibly 16-kD proteins are present at even the earliest stage isolated, that of young microspore. This is a stage at which the exine is rapidly synthesized. The other exine-associated proteins present in mature pollen are not as apparent in early developmental stages.

DISCUSSION

Although pollen wall development has been well studied at the cytological level, the biochemical aspects of this process are not understood. Recent success in obtaining antibodies to exines should be helpful in the initiation of molecular studies (27, 28). Our approach has been to purify exines from maize microspores and pollen and to analyze the proteins tightly associated with these structures. We have demonstrated that we are able to prepare highly purified pollen walls from several morphologically distinct developmental stages of pollen development. There are seven abundant proteins associated with mature maize pollen exines, which make up 0.2 to 0.3% of the total maize pollen protein. Many changes in gel migration are observed after chemical deglycosylation of exine fractions; more definitive studies on the glycosylation state of the exine-associated proteins are in progress.

A 25-kD protein in this group contains hydroxyproline, an amino acid associated with plant primary cell wall proteins, including extensins and AGPs (4). Recently, evidence for

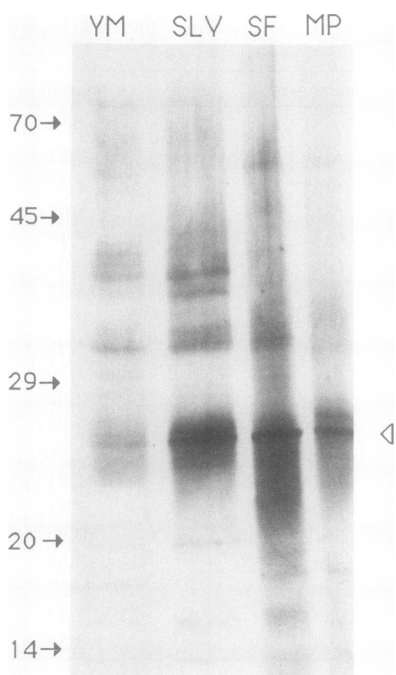


Figure 5. SDS-PAGE of final exine fractions from developmentally staged microspores and pollen. Fifty micrograms of protein were loaded in each lane. YM, Young microspores; SLV, single large vacuole microspores; SF, starch-filled young pollen; MP, mature pollen. Open arrow to right indicates position of the 25-kD exine-associated protein.

structural cell wall proteins in graminaceous monocots has been obtained. Two maize hydroxyproline-rich proteins have been purified: a threonine-rich one purified from maize suspension culture cells (15, 16) and another from maize pericarp (13). A maize coleoptile cDNA has been identified that encodes a cell wall protein identical or related to these purified proteins, thought to be in the extensin family (29).

The amino acid composition of the 25-kD protein strongly implies that it belongs in the AGP class of hydroxyproline-rich proteins (1, 8, 9, 30), given that it not only contains this unusual amino acid, but is also rich in serine and glycine and poor in lysine. The expression of AGPs is developmentally regulated in both vegetative (17) and reproductive (21) plant tissues, and these glycoproteins have been proposed to mediate important cell-cell interactions during development. Further studies on the 25-kD exine-associated protein are in progress to determine its possible role in pollen wall biosynthesis and its structural relationship to the hydroxyproline-rich primary cell wall proteins.

Some clues as to the function of these proteins may be derived from developmental studies. As discussed above, the microspores elaborate an extracellular structure that determines the pattern of pollen wall formation still enclosed in a special callose wall. This fibrillar structure, consisting of both carbohydrates and proteins, has been called the glycocalyx (23) or the primexine (11). Proteins detected very early in microspore development may therefore play a role in exine pattern formation.

After dissolution of the callose wall, sporopollenin is rapidly deposited on the microspore surface. It is thought that the sporophytic tapetal cells surrounding the developing microspores secrete sporopollenin precursors into the anther locule, where these are polymerized onto the glycocalyx template on the microspore surface (20). Exine-associated proteins first detected at this stage may be involved in sporopollenin polymerization or cross-linking during pollen wall growth. The elucidation of the cellular site and precise timing of exine-associated protein biosynthesis during pollen development lies in future studies.

ACKNOWLEDGMENTS

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