

Steryl Esters in the Elaioplasts of the Tapetum in Developing *Brassica* Anthers and Their Recovery on the Pollen Surface

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ABSTRACT: The tapetum cells in the developing anthers of *Brassica napus* contained abundant elaioplasts, which had few thylakoid membranes but were packed with globuli of neutral esters. Of the neutral esters, the major ester group possessed mainly 24-methylenecholesterol, 31-norcycloartenol, 24-dehydropollinastanol, and pollinastanol esterified to 18:3 and other unsaturated and saturated fatty-acyl moieties. The minor ester group had a dominant component tentatively identified as 12-dehydrolupeol esterified to mostly 18:0, 16:0, and 20:0 fatty-acyl moieties. The elaioplasts also contained a high proportion (16% w/w of total lipids) of monogalactosyldiacylglycerols (MGDG). This is the first report of plastids having steryl esters as the predominant lipids. We propose that the globuli contain steryl esters and are stabilized by surface MGDG and structural proteins. The tapetosomes, the other abundant lipid-containing organelles in the tapetum, possessed triacylglycerols (TAG) as the predominant lipids. At a late stage of anther development, the minor group of neutral esters and MGDG of the elaioplasts, as well as the TAG of the tapetosomes, were degraded. Steryl esters similar to those of the elaioplasts were recovered from the pollen surface and were the major lipids of the pollen coat. The pollen coat steryl esters and proteins could be extracted with moderately polar or nonpolar solvents. These proteins, which were mostly fragments of oleosins derived from the tapetosomes, had a high proportion of lysine (13 mol %). The possible functions of the steryl esters and the proteins on the pollen surface are discussed.

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Pollen is produced in the locules (pollen sacs) in the anthers of flowers (1,2). The maturing pollen grains interact with the tapetum, which consists of one layer of metabolically active cells enclosing the locule. The tapetum serves several important roles (2). Throughout the maturation process, the tapetum regulates the flow of nutrients and secretes enzymes to the locule for pollen maturation. At a late stage of anther development, the

tapetum cells lyse and discharge molecules onto the surface of the pollen, forming the pollen coat. The pollen coat consists of lipids, proteins, and small molecules such as sugars. The pollen coat may play diverse physiological roles, such as protecting the pollen from solar radiation, giving the pollen color, preventing water loss from the pollen, attracting insect pollinators, acting as an adhesive to affix the pollen together and to the insect pollinators and the stigma surface, aiding hydration and thus the initial germination of the pollen, and exerting self-compatibility and incompatibility (2).

The constituents of the pollen coat of most species are largely unknown, but those of the pollen coat of *Brassica* species have been studied (3–6). In *Brassica*, the pollen coat can be extracted from the pollen with an organic solvent such as cyclohexane or diethyl ether without apparent damage to the pollen protoplast. The coat consists of neutral esters as the major lipid constituents, and its predominant proteins are specifically fragmented oleosins. The oleosins belong to the family of proteins described earlier as abundant structural proteins on the storage oil bodies in seeds (7). The neutral esters and oleosin fragments that constitute the pollen coat are produced in the tapetum cells. At a late stage of anther development immediately before lysis of the tapetum, the tapetum cells contain two dominant organelles. Both organelles are spherical and about 3 μm in diameter (8,9). One of the organelles is a novel lipid particle termed tapetosome, which does not have an enclosing membrane and contains patches of triacylglycerols (TAG) situated among densely packed vesicles (6). The other organelle is the elaioplast, which is packed with globuli of neutral esters. Whereas the predominant proteins of the tapetosomes are oleosins, those of the elaioplasts include lipid-associated structural proteins (10), presumably located on the surface of the globuli. Just before or shortly after lysis of the tapetum cells, some of the constituents of the two organelles are degraded, whereas the other constituents remain unchanged or are modified, and are released onto the surface of the maturing pollen (4–6). These latter constituents include fragmented oleosins of the tapetosomes and the neutral esters of the elaioplasts.

We here report that the abundant neutral lipids in the elaioplasts are steryl esters. This is the first report of plastids contain-

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Abbreviations: APCI, atmospheric pressure chemical ionization; EI, electron impact; FAME, fatty acid methyl ester; GC, gas chromatography; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MGDG, monogalactosyldiacylglycerols; MS, mass spectroscopy; TAG, triacylglycerols.

ing steryl esters as the predominant lipids. Also, we demonstrate the selective recovery of the lipids and proteins of the tapetum elaioplasts and tapetosomes on the pollen surface.

EXPERIMENTAL PROCEDURES

Plant materials. *Brassica napus* L. var. Westar seeds were obtained from Calgene Inc. (Davis, CA) and used to produce flowering plants in a greenhouse maintained at 26.5/18.5°C, 14/10 h day/night cycle. The florets were divided into five developmental stages according to the following criteria (10): at stage 1, the florets were 2 mm long or less, and more than half of the microspores (i.e., the maturing pollen) were in a tetrad condition; at stage 2, the florets were 2–3 mm long, and all the microspores were solitary; at stage 3, the florets were 3–4 mm long, and the tapetum cells were unlysed and filled with organelles; at stage 4, the florets were 4–5 mm long, and the tapetum cells had just lysed; and at stage 5, the florets were 5–6 mm long, and the microspores were almost mature as pollen. Mature pollen was collected from flowers that had opened on the same day.

Isolation of tapetosomes and elaioplasts from the florets. All the solutions described in this paragraph contained 0.05 M HEPES-NaOH, pH 7.5. The florets of stage-3 anthers were finely minced with a razor blade in a petri dish containing a grinding medium of 0.8 M sucrose (125 florets of 3.1 g per 8 mL) (6). Light microscopy revealed that chopping broke most of the sporophytic floral tissues, especially the tapetum, but left the gametophytic microspores intact. These microspores were stained positively with the vital dye fluorescein diacetate (observed by light microscopy). The homogenate was filtered through a layer of Nitex cloth (20 × 20 μm pore size), which removed the microspores. The filtrate (4 mL) was placed in a 17-mL centrifuge tube (6). For the preparation of a mixture of the two organelles (tapetosomes and elaioplasts) in a one-step density gradient, a solution of 0.4 M sucrose solution was layered on top of the filtrate. For the separation of the two organelles in a three-step density gradient, successive layers of 4 mL each of 0.4, 0.2, and 0 M sucrose solutions were placed on top of the filtrate. The tube was centrifuged at 9,000 rpm in a Beckman SW 28.1 rotor for 2 h. In the one-step density gradient, the low-density organelle fraction floating on top was collected with a pipet. In the three-step density gradient, the two visible organelle fractions banding at the interfaces between 0.4 and 0.2 M (tapetosomes), and 0.2 and 0 M (elaioplasts) sucrose solutions were collected from the bottom of the centrifuge tube after a hole was punctured through the tube with a needle.

Preparation of surface fractions from the mature pollen. The procedure followed that described earlier (6). Mature pollen was mixed with 2 vol of diethyl ether (or other solvents, to be described in the Results and Discussion section) for 1 min by repeated inversion in a capped test tube. The pollen was separated from the solvent by centrifugation for 10 min at 800 × g. The supernatant was retained and evaporated under vacuum.

Analyses of the proteins in the pollen surface fraction. The amino acid composition was analyzed by the Protein Structure Facility at the University of Iowa, Iowa City, IA. The polypeptide composition was analyzed by 12.5% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2.5 h at 100 V (6). After electrophoresis, the gel was stained with Coomassie blue and then destained.

Extraction of lipids from the various samples. Lipids in the various samples were extracted with chloroform/methanol (2:1, vol/vol) using the procedure of Folch *et al.* (11).

Separation of neutral lipids in pollen coat extracted with different solvents. Lipids in chloroform/methanol (2:1, vol/vol) were applied to thin-layer chromatography plates (silica gel 60A, Whatman Inc., Clifton, NJ). The plates were developed in hexane/diethyl ether/acetic acid (80:20:2, by vol), and then charred with sulfuric acid.

Quantitative analyses of the neutral and polar lipids. Non-polar lipids were quantitatively analyzed using a high-performance liquid chromatography (HPLC) system that consisted of a Hewlett-Packard (Avondale, PA) Model 1050 Quaternary Solvent HPLC and autosampler, and an Alltech-Varex (Deerfield, IL) Mark III Evaporative Light-Scattering Detector. The column was a LiChrosorb Diol column (Chrompack, 3 × 100 mm), and the flow rate was 0.5 mL/min. The solvents were A, hexane/acetic acid, 1000:0.3, vol/vol; and B, hexane/isopropanol, 100:1, vol/vol. (Both were mixed fresh daily to eliminate variability caused by evaporation and/or absorption of moisture). The linear gradient timetable was as follows: at 0 min, 100:0; at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100:0 (%A/%B, respectively), with a total run time of 60 min. Polar lipids (glycolipids and phospholipids) were quantitatively analyzed using an HPLC-evaporative light-scattering detection system as previously described (12).

Analysis of alcohols from E1 and fatty acids from E1 and E2. Lipids in HPLC fractions E1 (defined as lipids with a retention time of 2.3 min) and E2 (retention time of 3.4 min) were saponified with 1 N KOH in 80% ethanol (reflux 1 h at 90°C). After cooling, the alcohols were extracted with hexane, the ethanolic phase was acidified with 6 N HCl, and free fatty acids were recovered by a second hexane extraction. The free fatty acids were then derivatized to methyl esters (FAME) with 14% boron trifluoride in methanol (20 min at 70°C sealed under N₂) for gas-liquid chromatography (GLC) analysis. Prior to GLC and gas chromatography-mass spectrometry (GC-MS) analyses, alcohol and FAME fractions were partially purified by hexane/diethyl ether step gradient elution from Pasteur pipet silicic acid columns (13). Free sterols derived from the steryl esters in HPLC fraction E1 were quantified by capillary GLC-flame-ionization detection on a 30 m × 0.25 mm i.d., 0.25-μm SPB-1 film column (Supelco, Bellefonte, PA) as described previously (14). Sterols were identified on the basis of their retention times relative to authentic standards and by GC-MS followed by comparison of their electron impact (EI) mass spectra with those from standards, from the literature, and/or from the NIST (National Institute of Standards and Technology, e-

mail: <http://webbook.nist.gov>) spectral library. FAME derived from fraction E1 and E2 lipids were quantified by capillary GLC–flame-ionization detection, with separation on a 15 m × 0.25 mm i.d., 0.20- μ m SP-2330 film column (Supelco) as previously described (15). Identification was based on FAME retention times relative to authentic standards, with confirmation by electron impact (EI) GC–MS. Electron ionization spectra (70 eV, source temperature 180°C) of free sterols and FAME were collected from m/z 40 to 500 using a Hewlett-Packard 6890 series gas chromatograph and mass-selective detector fitted with a 30 m × 0.25 mm i.d., 0.25- μ m SPB-1 film capillary column. For sterols, the column oven was programmed to increase from 200 to 240°C at 10°C per min, followed by an increase from 240 to 260°C at 1°C per min, with a final 12 min at 260°C. For FAME, the column oven program included an initial rise from 100 to 150°C at 10°C per min, followed by a further increase from 150 to 200°C at 2°C per min and a final 10 min at 200°C.

Analysis of E2 and alcohols from E2. The lipids were analyzed by HPLC–MS–atmospheric pressure chemical ionization (APCI). The HPLC system for separation of nonpolar lipids described in an earlier section was connected to a Hewlett-Packard Model 5989A mass spectrometer, with an APCI interface operated in the positive ion mode. The ion source was at 100°C, and the instrument scanned from 50–1000 m/z at 2.0 s/scan.

RESULTS AND DISCUSSION

Lipid composition of the florets and the subcellular fractions. Lipids extracted from the whole florets (omitting the maturing pollen) and the various organelle fractions were analyzed

via one HPLC system for the nonpolar lipids and another HPLC system for the polar lipids (Table 1). The abundant lipids in all the samples were neutral lipids and glycolipids. Those in the whole florets included a major (E1, about 80%) and a minor (E2, about 20%) group of fatty-acyl esters, TAG, and monogalactosyldiacylglycerol (MGDG). E1 and E2 ester fractions were separated in the nonpolar HPLC system, with retention times of 2.3 and 3.4 min, respectively. Whereas the E1 esters co-chromatographed with steryl-fatty acyl ester standards, no known standards co-chromatographed with the E2 ester. The abundant lipids and the minor lipids of the whole florets were also present in roughly the same proportion in the two-organelle fraction, which was a mixture of approximately equal amounts of the elaioplasts and the tapetosomes of the tapetum. The similarity is expected because the tapetum cells contained the two organelles as the predominant constituents and were the most metabolically active cells in the anthers (other than the maturing pollen).

The isolated elaioplast fraction contained E1, E2, and MGDG as the major neutral lipids, whereas the isolated tapetosome fraction contained mostly TAG. Using the neutral esters and TAG as markers of the two organelles (6), we estimate that the elaioplast fraction contained little contaminating tapetosomes, whereas the tapetosome fraction had some (about 15% w/w) contaminating elaioplasts. This pattern of cross contamination is expected because the elaioplasts moved to a lower density region than the tapetosomes during flotation gradient centrifugation, resulting in a relatively pure elaioplast fraction and a less pure tapetosome fraction containing some tailing elaioplasts. The elaioplast fraction possessed a high amount of MGDG (about 16% w/w of total lipids). Since the elaioplasts have minimal envelope and thy-

TABLE 1
Quantitative Analysis of Lipid Classes in Different Extracts of the *Brassica napus* Florets and the Pollen Surface^a

Lipids	Whole florets	Two-organelle fraction	Elaioplasts	Tapetosomes	Pollen surface
	μ g lipid class/mg total lipids ^b				
E1	219	201	347	52	298
E2 ^c	68	61	111	26	10
TAG	322	352	20	539	11
FFA	23	9	14	Trace	7
St	35	17	38	18	42
MGDG ^d	96	122	156	24	Trace
DGDG	29	3	3	5	13
PE	15	9	10	48	Trace
Lyso-PE	4	15	15	3	38
PI	5	2	2	11	0
PA	3	2	1	13	3
PC	18	11	32	42	4
Lyso-PC	9	5	27	4	4

^aThe two-organelle fraction was a mixture of approximately equal amounts of the elaioplasts and tapetosomes.

^bLipids that were not accounted for in each sample were very nonpolar lipids (e.g., squalene, β -carotene, or other hydrocarbons) that eluted as a single peak in the void volume (1.5 min) by nonpolar high-performance liquid chromatography (HPLC).

^cFor unknown reasons, some preparations of the whole floret, the two-organelle, and the elaioplast fractions contained higher amounts of E2 relative to E1 (about 50–80% rather than the indicated 30–32%).

^dThe peak fraction of monogalactosyldiacylglycerols (MGDG) resolved by HPLC might contain some steryl glycoside and glucocerebroside. TAG, triacylglycerols; FFA, free fatty acids; ST, sterols, DGDG, digalactosyldiacylglycerols; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine. For explanation of E1 and E2 see the Experimental Procedures section.

lakoid membranes and are packed with small (about 0.2 μm in diameter) globuli (6), which presumably are made up of the neutral esters, it is likely that the MGDG is present on the surface of the globuli. The tapetosomes contained abundant internal vesicles of presumably membranous nature (6), and their predominant phospholipids were phosphatidylcholine and phosphatidylethanolamine. The lipids on the pollen surface contained mostly E1 and had little E2 and TAG.

All the fractions (Table 1) contained very nonpolar lipids, which were eluted with the void volume (1.5 min.) in the nonpolar HPLC system. These represented a complex mixture of various lipids, such as squalene, β -carotene, or other hydrocarbons. No attempts were made to further separate and characterize them.

Characterization of the major neutral esters in the elaioplasts. The major group of neutral esters, E1, and the minor group of neutral esters, E2, were characterized further after saponification. The acyl moieties in E1 and E2 were very different (Table 2). In E1, 50% of the acyl moieties were 18:3, 18:2, and 18:1, and the remaining 50% were the saturated

acids 16:0, 14:0, 18:0, and 20:0. In E2, only 6% of the acyl moieties were unsaturated, and the saturated moieties included mostly 18:0, and 16:0.

The saponified E1 contained a mixture of at least 16 sterols, 14 of which (accounting for about 98% of the total) were identified and quantified by GLC and GC-MS (Table 3). Of these sterols, the common desmethyl sterols typically found in plant cell membranes (i.e., sitosterol, campesterol, stigmasterol, and cholesterol) constituted less than 16%. These desmethyl sterols are likely to represent the constitutive components of the limited envelope and internal membranes (6). The four most abundant components included 24-methylenecholesterol and three unusual 9 β ,19-cyclopropane sterols (pollinastanol, 24-dehydropollinastanol, and 31-norcycloartenol) derived from a branch pathway of cycloartenol metabolism. They are likely the major constituents of the globuli in the elaioplasts (6,9). These steryl esters are known to be present in pollen from various species (16).

We have so far been unable to identify definitely the predominant alcohol moiety of E2. For both the intact E2 esters and the major alcohol moiety recovered after saponification, the soft-ionization achieved with APCI-MS in the positive mode yielded an intense peak at m/z 407. In assuming that this high-mass fragment represented the molecular ion of the E2 alcohol minus water plus a proton ($M^+ - 18 + 1$), which is often observed in mass spectra of sterols and triterpenols, the alcohol should have a molecular weight of 424. Although the EI GC-MS spectrum of the underivatized E2 alcohol showed only a small number of minor peaks above m/z 200, the highest mass ion was m/z 424, thus supporting the molecular weight proposed on the basis of the APCI-MS data. The pattern of ions $\leq m/z$ 203 obtained with EI GC-MS was similar to that reported for lupeol, a triterpenol with a molecular

TABLE 2
Fatty-Acyl Composition of the Elaioplast E1 and E2 Neutral Esters

Acyl moiety	E1 % (wt/wt) of the fatty-acyl methyl esters	E2
14:0	11.6	6.7
16:0	17.8	37.8
18:0	10.7	35.9
18:1	3.9	2.3
18:2	11.8	2.6
18:3	34.9	1.7
20:0	8.7	10.0
22:0	0.6	1.3
24:0	0	0.9
26:0	0.9	0.8

TABLE 3
Sterol Composition of the Elaioplast E1 Steryl Esters Isolated by HPLC

Phytosterol	Retention time ^a	Molecular mass	Formula	Area %
Cholesterol ^b	1.00	386	C ₂₇ H ₄₆ O	1.1
Lathosterol	1.09	386	C ₂₇ H ₄₆ O	0.9
Pollinastanol	1.12	400	C ₂₈ H ₄₈ O	11.5
24-Methylenecholesterol	1.21	398	C ₂₈ H ₄₆ O	23.2
24-Dehydropollinastanol	1.22	398	C ₂₈ H ₄₆ O	12.5
Campesterol	1.24	400	C ₂₈ H ₄₈ O	5.3
31-Norcycloartenol	1.29	414	C ₂₉ H ₅₀ O	1.2
Stigmasterol	1.33	412	C ₂₉ H ₄₈ O	0.7
24-Methylenepollinastanol	1.35	412	C ₂₉ H ₄₈ O	2.1
31-Norcycloartenol	1.38	412	C ₂₉ H ₄₈ O	20.9
α -Sitosterol	1.47	414	C ₂₉ H ₅₀ O	8.7
Isofucosterol	1.51	412	C ₂₉ H ₄₈ O	8.3
Cycloeucaenol	1.53	426	C ₃₀ H ₅₀ O	0.7
Cycloartenol	1.60	426	C ₃₀ H ₅₀ O	0.9
Others (unidentified)	—	—	—	2.0

^aGas chromatography retention time relative to cholesterol.

^bSterol nomenclature: cholesterol, cholest-5-en-3 β -ol; lathosterol, cholest-7-en-3 α -ol; pollinastanol, 14 α -methyl-9 β ,19-cyclo-5 α -cholestan-3 β -ol; 24-methylenecholesterol, ergosta-5,24(28)-dien-3 β -ol; 24-dehydropollinastanol, 14 α -methyl-9 β ,19-cyclo-5 α -cholest-24-en-3 β -ol; campesterol, 24[R]-ergost-5-en-3 β -ol; 31-norcycloartenol, 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -cholestan-3 β -ol; stigmasterol, stigmasta-5,22-dien-3 β -ol; 24-methylene-pollinastanol, 4 α -methyl-9 β ,19-cyclo-5 α -ergost-24(28)-en-3 β -ol; 31-norcycloartenol, 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -cholest-24-en-3 α -ol; β -sitosterol, stigmast-5 α -en-3 β -ol; isofucosterol, stigmasta-5,24(28)-dien-3 β -ol; cycloeucaenol, 4 α ,14 α -dimethyl-9 β ,19-cyclo-5 α -ergost-24(28)-en-3 β -ol; cycloartenol, 4,4,14 α -trimethyl-9 α ,19-cyclo-5 α -cholest-24-en-3 β -ol.

weight of 426 (the base ion, however was m/z 81 rather than 43 as in lupeol). These limited data are consistent with the E2 alcohol being the triterpenol 12-dehydrolupeol, which has been reported to occur as an acetate ester in *Hemidesmus indicus* (17). Further analysis of this alcohol is required for unequivocal identification.

Green or nongreen plastids containing steryl esters as the predominant lipids have not been previously reported. The best-studied nongreen plastids containing high amounts of specific lipids are the chromoplasts, which possess abundant pigments (18). These pigments, mainly carotenoids, are present in globuli in the stroma or fibril structures associated with the thylakoids. The globuli in chromoplasts of some species also contain a high percentage of TAG. The globuli or the fibrils are covered with special structural proteins that are highly conserved in amino acid sequences among species (19). In comparison with these chromoplasts and other plastids, the *Brassica* tapetum elaioplasts are unique. They contain minimal internal membranes and stroma and are packed with lipid globuli. These globuli contain steryl esters and are presumably covered with MGDG (Table 1) and structural proteins similar to those in chromoplasts (10). Elaioplasts of *in situ* morphology similar to that of *Brassica* elaioplasts have been observed in the tapetum of diverse species (2), indicating that they may also have similar lipid and protein compositions.

Developmental changes of the major neutral lipids in the florets. Changes during floret maturation in the contents of the major neutral lipids in the sporophytic anthers, and ultimately on the pollen surface, were determined (Fig. 1). Elaioplast E1 (steryl esters) accumulated during maturation from stage 1 to stage 5. Electron microscopic observations (8,9) showed that at stage 5, the tapetum cells started to lyse, and the elaioplasts or the released globuli were transferred to the surface of the maturing pollen. Steryl esters similar to those of the elaioplasts were recovered quantitatively from the pollen surface (Fig. 1). The current biochemical analysis and the earlier electron microscopic observations strongly suggest that the steryl esters were transferred from the tapetum elaioplasts to the pollen surface. In contrast with the elaioplast E1 steryl esters, the elaioplast E2 (Fig. 1) and MGDG (Table 1), as well as the tapetosome TAG (Fig. 1), increased to a maximum at developmental stage 3 and then declined to minimal amounts on the pollen surface (Fig. 1). Thus, the pollen surface contained only E1 as the predominant lipid component (Fig. 1 and Table 1). The relatively high percentage of lysophosphatidylethanolamine in the pollen coat (Table 1) is indicative of the presence of phospholipase A2 in the anthers during development.

The physiological significance of the accumulation of E2 in the elaioplasts and TAG in the tapetosomes during the mid-maturation stage of the anthers and of their subsequent degradation rather than transfer to the pollen surface is unknown.

Extraction of lipids and proteins from the surface of the pollen. It was reported that the lipids and proteins on the surface of the pollen could be extracted with cyclohexane or diethyl ether without apparent damage to the pollen protoplasts (3,4,6).

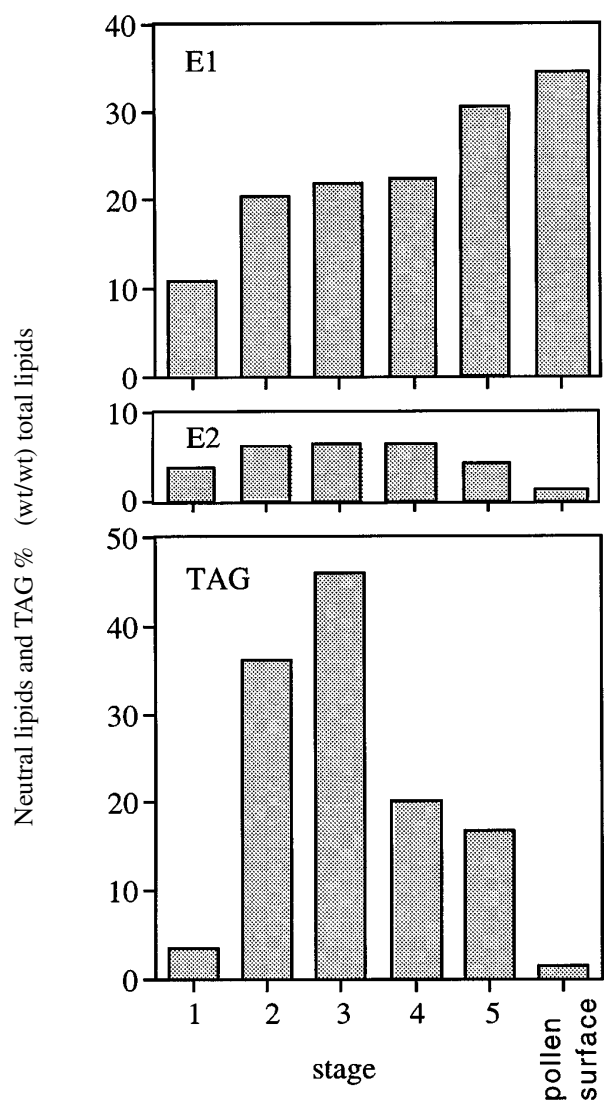


FIG. 1. Proportions of major neutral lipids in *Brassica napus* sporophytic florets at five stages of development and in the pollen coat. Data on the neutral esters E1 and E2 and triacylglycerols (TAG) are expressed as % wt/wt of total lipids. The amounts of total lipids in the florets at stages 1 to 5 and on the pollen surface were 1.01, 2.40, 2.20, 2.22, 2.28, and 2.29 mg per 15 florets, respectively.

In the present study, we found that all the neutral lipids and proteins of the pollen coat could be simultaneously extracted with nonpolar and moderately polar solvents (Fig. 2). They were extracted incompletely with methanol, and not at all with water. The pollen surface lipids shared some similarities with the total pollen lipids (Fig. 2). The total pollen lipids contained the pollen surface E1 and other neutral esters of the pollen interior (6), which comigrated with E1. They also contained TAG, which should be those in the storage lipid bodies in the cytoplasm. The pollen surface proteins were resolved into a pattern by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which differed from that of the total pollen proteins (Fig. 2).

Most of the polypeptides in the pollen coat had sizes in the ranges of 30–37 kDa and <14 kDa (Fig. 2). They largely repre-

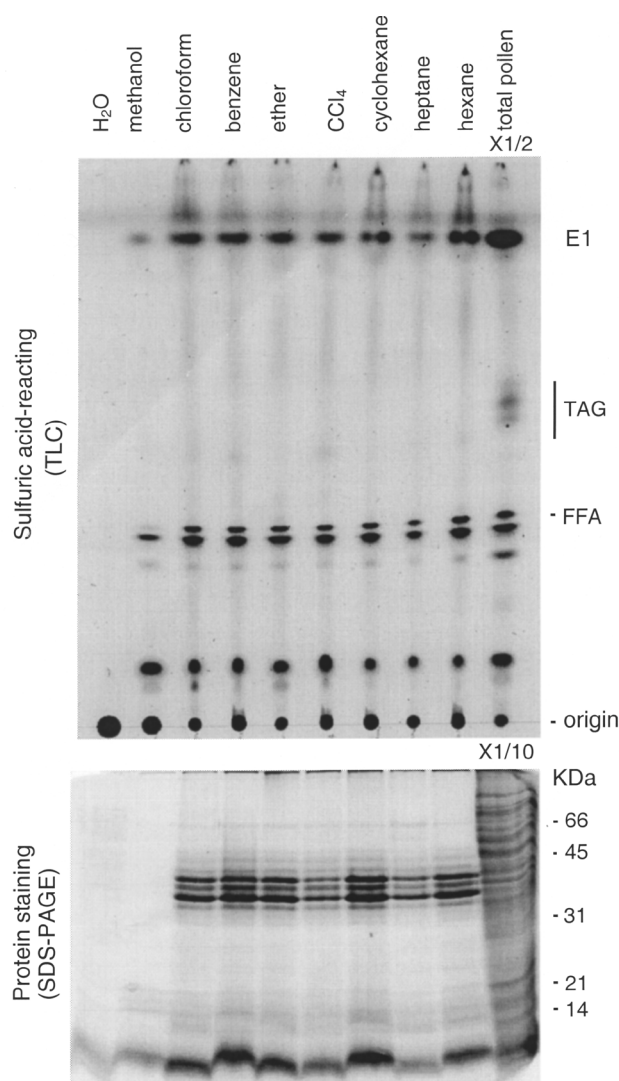


FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and thin-layer chromatography (TLC) of neutral lipids of pollen coat extracted from the pollen of *Brassica napus* by different solvents. Loading of extracts obtained with various solvents was arranged in the order of decreasing solvent polarity from left to right. Total pollen extract was prepared by grinding pollen in diethyl ether in glass beads with a mortar and pestle. A pollen-coat sample equivalent to an extract from 2 mg (TLC) or 3 mg (SDS-PAGE) of pollen, or total pollen extract from 1/2 of 2 mg (TLC) or 1/10 of 3 mg (SDS-PAGE) of pollen was loaded to each lane. Positions of markers for protein molecular weights and standard lipids are shown on the right. For other abbreviations see Figure 1.

sented specifically fragmented oleosins of the tapetosomes (4, 10). The amino acid sequences of these fragments, as predicted from their gene nucleotide sequences, indicate that they are very hydrophilic. Yet, these very hydrophilic polypeptides and the very hydrophobic steryl esters were extractable by moderately and strongly nonpolar solvents. These findings suggest that on the pollen surface, the neutral lipids of the elaioplasts and the polypeptides of the tapetosomes are mixed and emulsified with amphiphilic molecules, such as phospholipids and galactolipids (Table 1).

TABLE 4
Amino Acid Compositions of the *Brassica* Pollen Coat Proteins and the Two Major Oleosin Fragments Known to Be in the Pollen Coat^a

	Pollen coat	Fragment from 45-kDa oleosin	Fragment from 48-kDa oleosin
		mol%	
Ala	8.3	2.2	1.9
Cys	3.1	0	0
(Asx) asp	(7.8)	4.9	3.8
(Glx) glu	(8.0)	7.2	7.3
Phe	2.4	0.38	0.32
Gly	13.8	26.0	26.9
His	3.4	7.5	7.6
Ile	4.3	4.9	4.8
Lys	13.3	16.6	14.9
Leu	5.4	0.75	0.63
Met	2.6	2.3	2.9
(Asx) asn	(7.8)	1.9	2.2
Pro	6.1	2.6	3.5
(Glx) gln	(8.0)	0.38	0.63
Arg	3.6	0.38	0.63
Ser	11.5	17.3	18.7
Thr	4.1	3.8	1.9
Val	3.3	0.38	1.3
Try	0	0	0
Tyr	1.5	0.38	0.32

^aThe amino acid compositions of the oleosin fragments were deduced from their known amino acid sequences (4,10).

An analysis of the amino acid composition of the total proteins on the pollen surface revealed a very high proportion (13 mol%) of lysine (Table 4). This is a reflection of the high lysine content in the short repeated sequences of the two predominant oleosin fragments on the pollen surface (4,20).

The high-lysine proteins and the plentiful steryl esters are both essential nutrients of the pollinating bees and other insects. Whether these components make a substantial impact in increasing the population of the pollinating insects is unknown. Some minor constituents of the pollen coat are originated from the pollen gametophyte and involved in self-incompatibility (3). The steryl esters on the pollen surface could play one or more of the roles of the pollen coat described in the Introduction. In addition, they could act as feeding stimulants of insects (21) or hormonal stimulants of pollen germination and tube growth (22).

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