The Occurrence of Phenols as Degradation Products of Natural Sporopollenin – a Comparison with "Synthetic Sporopollenin"

Susanne Herminghaus, Sabine Gubatz, Sibylle Arendt, and Rolf Wiermann

Botanisches Institut der Universität Münster, Schloßgarten 3, D-4400 Münster, Bundesrepublik Deutschland

Z. Naturforsch. **43c**, 491–500 (1988); received December 23, 1987

Corylus avellana L., Betulaceae, Pollen, Sporopollenin, Phenols

1. A highly purified sporopollenin fraction from *Corylus avellana* pollen was obtained using a gentle method employing hydrolyzing enzymes (pronase, lipase, cellulase, amylase, cellulysin) and an exhaustive extraction using different solvents.

2. The sporopollenin fractions were degraded by potash-fusion and nitrobenzene oxidation and the low molecular decomposition products were analyzed by TLC and HPLC. The investigation centered solely on the proof of phenolic compounds.

3. The degradation by potash-fusion yielded *p*-hydroxybenzoic acid as a main component, while the degradation by nitrobenzene oxidation (NBO) resulted in the formation of *p*-hydroxybenzaldehyde as the main component. In addition phenolic components such as *p*-coumaric acid, ferulic acid, vanillin and vanillic acid were formed to different degrees by using NBO as degradation procedure.

4. A comparison of the products formed following degradation of *Corylus* sporopollenin and "synthetic sporopollenin" shows, that phenolic compounds, if they indeed occurred as degradation products of "synthetic sporopollenin", are generated only in extremely small quantities. It appears that, in contrast to several reports in the literature, phenols are integral constituents of natural sporopollenin. This view is supported by unpublished tracer experiments.

Introduction

The structure and biosynthetic pathway of sporopollenin are largely unknown. In 1971, Brooks and Shaw [1, 2] have hypothesized that sporopollenin is generated from carotenoids and carotenoid esters. As far as higher plants are concerned, this hypothesis has not been supported by unambiguous experiments, such as tracer studies.

Investigations with inhibitors of carotenoid biosynthesis have shown that an intact carotenoid metabolism is not essential for undisturbed sporopollenin accumulation; however an involvement of terpenoid metabolism in the sporopollenin biosynthesis could not be completely excluded [3, 4].

During the decomposition of a highly purified sporopollenin fraction of *Pinus* pollen various phenolic compounds, some in considerable quantities, were released. Their appearance shows that phenols are an integral part in *Pinus* sporopollenin [5]. To what extent these results may be applied to other species, particularly angiosperms, is as yet unclear. Elemental analyses [6] and histochemical investigations [7, 8] suggest that sporopollenins from different sources have varying compositions.

The pollen from an angiosperm species was investigated for comparative purposes. Therefore a sporopollenin fraction from *Corylus* pollen was degraded by potash-fusion and nitrobenzene oxidation, and the phenolic compounds of the resulting degradation products were analyzed. The results from naturally occurring sporopollenin were compared with those from a "synthetic sporopollenin". An important prerequisite for the examination was the isolation and purification of natural sporopollenin. Instead of using the conventional aggressive methods (acetolysis [1]; phosphoric acid [10]) a gentle procedure using various hydrolytic enzymes was developed.

Materials and Methods

Plant material

Hazelnut pollen (*Corylus avellana* L.) was collected from the area around Münster (F.R.G.) and stored at −20 °C immediately following harvest. The material was freeze-dried before use.

Reprint requests to Prof. Dr. R. Wiermann.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0700–0491 $ 01.30/0

Isolation and enrichment of highly purified sporopollenin

The rupture of the pollen was achieved by stirring with a magnetic rod for 19 h at 6 °C followed by ultrasonic treatment. Using a glycerol gradient (for details see [11]) large pollen fragments of relatively similar size could be concentrated in a distinct band. The material from this fraction was repeatedly washed with Tris/HCl buffer (0.2 M, pH 8.8) and distilled water, and was finally treated sequentially with different enzymes, in order to break down and eliminate non-exine components (Fig. 1; Table I).

1. The pronase incubation mixture comprised:
   wall fragments (300 mg freeze-dried material),
   9.8 ml sodium phosphate buffer (0.066 M, pH 7.4),
   0.1 ml streptomycin (10 mg/ml distilled water),
   0.1 ml pronase (3.34 mg/ml sodium phosphate buffer).

   The material was incubated 5 times for 1 h at 40 °C by gentle stirring (Fig. 2a). Following each incubation it was washed twice with sodium phosphate buffer. After each incubation and washing the pollen fragments were centrifuged and, as a control for the efficiency of each enzyme treatment and washing step the protein content of the supernatant was determined (Method according to Bradford).

2. The lipase incubation medium comprised:
   wall fragments (residual sediment after incubation with pronase),
   4.4 ml Tris/HCl buffer (0.2 M, pH 8.0),
   0.1 ml streptomycin (10 mg/ml distilled water),
   0.5 ml $CaCl_2$ (1 M),
   5 ml lipase (2 mg/ml Tris/HCl buffer).

   The pollen fragments were incubated twice (each step 30 min) at 37 °C. They were then washed in a 1% taurocholate solution, followed by washing twice with Tris/HCl buffer. Determination of hydrolysis products was not carried out.

3. "Onozuka" R-10 cellulase incubation medium:
   wall fragments (remaining sediment after incubation with lipase),
   9.3 ml sodium acetate buffer (0.17 M, pH 5.0),
   0.1 ml streptomycin (10 mg/ml distilled water),
   0.1 ml ampicillin (6.25 mg/ml distilled water),
   0.5 ml purified enzyme solution (see below).

   After the final washing procedure which followed the lipase incubation the samples were treated with sodium acetate buffer (0.17 M, pH 5.0). The sugar content (control value, Fig. 2b) was determined in the supernatant after centrifugation of the wall fragments. The samples were incubated afterwards with cellulase 17 times, each for 2 h, at 40 °C. Following each incubation step the wall fraction was washed with buffer (2–4 times) until the sugar content in the supernatants was negligible. Determination of sugar content was carried out according to the anthrone method; the data are presented as glucose equivalents. Some of the supernatants of the 17 cellulase treatments were analyzed using a gas chromatograph and the sugars released were determined.

   The commercial cellulase preparation contained such a high amount of sugar components that it was impossible to determine the hydrolysis products in the supernatants quantitatively. The enzyme preparation was therefore purified before use as follows: 600 mg cellulase "Onozuka" R 10 was stirred in 30 ml sodium acetate buffer (0.17 M, pH 5.0) for 10 to 15 min at 4 °C. Insoluble components were centrifuged and the sugar and soluble protein contents were determined in the supernatants (Fig. 3(1)).

   After $(NH_4)_2SO_4$-precipitation (80% saturation) of the protein the purification effect of this step was investigated by determination of sugar and protein contents in the supernatants (Fig. 3(2)).

   The precipitate was dissolved in 4 ml sodium acetate buffer and loaded onto a trisacryl column. Protein and sugar concentrations were measured in the protein peak fractions. The ratio of the two components was relatively constant in the different fractions implying that the remaining sugar was bound to the protein (Fig. 3(3)).

   In control experiments the amount of sugar associated with the purified enzyme was determined. Carbohydrate determination after enzyme treatments were corrected for these blank values. The antibiotic reagents did not interfere with the anthrone sugar determination.

4. Amylase incubation medium:
   wall fragments (residual sediment after cellulase incubation);
   8.8 ml potassium phosphate buffer (0.02 M potassium phosphate, 0.01 M sodium chloride, pH 6.9),
   0.1 ml streptomycin sulfate (10 mg/ml distilled water),
   0.1 ml ampicillin (0.25 mg/ml distilled water),
   1.0 ml purified enzyme solution (see below).

   The wall fraction was treated 5 times with amylase (2 h each at 40 °C). Only negligible amounts of

hydrolysis products were produced (Fig. 2c). The sediment was washed exhaustively with buffer after each treatment.

The enzyme amylase had to be purified as well as cellulase because of interference with sugar determinations. For this 600 mg of commercial amylase was stirred in 120 ml potassium phosphate buffer (see above) and the insoluble components were precipitated by centrifugation. The subsequent treatment of the enzyme, and the protein and sugar determinations were carried out described for cellulase. The purification effects were comparable to those shown in Fig. 3.

5. Cellulysin incubation medium:

The reaction mixture had the same composition as was described for the cellulase treatment. An incubation for 2 h at 40 °C was repeated 4 times (Fig. 2d). The purification of the commercial enzyme, which also contained sugars, was carried out as for cellulase, with the exception that 0.1 M sodium acetate buffer (pH 5.0) was used.

The exhaustive extraction with solvents

Pollen fragments were treated with distilled methanol following enzyme incubation and 2 washing steps with distilled water in order to eliminate non-covalently bound substances located on the surface and/or inside the pollen walls. The residual pollen fragments, which had been enzymatically treated and freeze-dried were subsequently extracted with 15 ml of distilled methanol by gentle stirring. The total extraction lasted 143 days. At first the fragments were centrifuged and the methanol renewed every 30 min, subsequently the methanol was changed approximately every 100 h. In order to extract more efficiently, the temperature was raised from 20 °C (the first 300 h) to 40 °C (the next 100 h) and finally to 50 °C. In order to facilitate sedimentation of the pollen fragments diethylether, equivalent to 1/10 of the extraction volume, was added to the sample before centrifugation 300 h after the beginning of the extraction. In addition to methanol other solvents, for example ethyleneglycolmonomethylether, were used.

"Synthetic sporopollenin"

A polymer synthetized from β-carotine designated "synthetic sporopollenin", was a kind gift from Dr. Geisert, Mainz.

Chemical degradation of the sporopollenin fraction

The potash-fusion and nitrobenzene oxidation (NBO) were carried out as described elsewhere [5]. In contrast to the published method NBO was performed in *sealed* steel tubes.

TLC/HPLC/GC

TLC: Silicagel plates (Merck, Darmstadt, F.R.G.) were developed with chloroform as a solvent (= TLC-1), cellulose plates (Merck) were run with 3% acetic acid (= TLC-2) and toluene-methanol-acetic acid as solvents (TLC-3). The products were detected before and after treatment with ammonia as well as by spraying with hydrazine sulfate (test for phenolic aldehydes [12]) or *p*-nitrobenzene diazonium tetrafluoroborate. In the latter case the dried disks were treated subsequently with Na_2CO_3 (test for phenolic acids [12]).

HPLC: Gilson, system 42

A discontinuous gradient of acetonitrile (85% eluent A) and acetic acid (1% eluent B) was used. Separation was accomplished on columns (Li Chrosorb RP8, Merck; Spherisorb 5 ODS III, Kontron) by elution with the following gradients: 0–15 min: A = 10%, 15–20 min: A = 50%, 20–21 min: A = 70%, 21–23 min: A = 100%, 23–25 min: A = 10%.

GC: Varian 1400, 25 m glass capillary column packed with OV-101 (Macherey and Nagel, Düren; F.R.G.).

GC: Individual samples obtained after cellulase treatment as well as from the control assay were deproteinized by the method of [13], loaded on serial cation- and anion exchanger (Dowex resin), and were silylized afterwards. Reference substances were treated identically.

The GC investigations were complemented by GC-MS analysis. Dr. Austenfeld (Institut für Angewandte Botanik, Münster, F.R.G.) and Dr. Luftmann (Institut für Organische Chemie, Münster) provided valuable support and advice for the GC and GC-MS analyses.

Sugar and protein determination

Sugar determination was carried out according to the anthrone sulfuric acid method [14]. The sugars released were quantified using glucose as a standard.

Protein determination was carried out according to the method of Bradford [15] with the modification of Read and Northcote [16] using ovalbumin as a standard.

Results

Isolation and purification of a sporopollenin fraction

In order to investigate sporopollenin degradation, it is necessary to obtain a highly purified exine fraction (sporopollenin), which has to be free from intine components and non-covalently bound material.

To obtain sporopollenin, pollen grains were ruptured mechanically followed by fractionation of the pollen fragments on a glycerol gradient (see [11]) and treatment with different hydrolytic enzymes and organic solvents.

The efficiency and the course of each treatment with the exception of lipase incubation was monitored by quantitative determination of the hydrolytic products. In the case of the lipase treatment it was shown that the enzyme is active in the presence of pollen fragments.

The treatments were repeated until no hydrolytic products could be detected with the methods employed (Fig. 2, a-d). The majority of hydrolytic products was released after treatment with cellulase (Fig. 2b). Quantitative analysis of some of the supernatants after cellulase incubation using GC and GC-

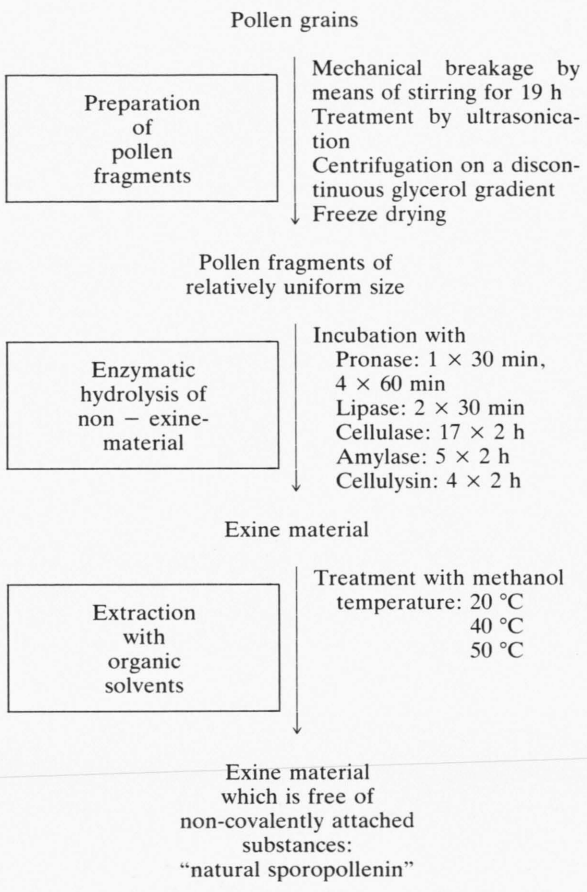


Fig. 1. Scheme of the isolation and purification procedure of sporopollenin from *Corylus* pollen.

Table I. Sequence of enzymatic treatments of pollen fragments: incubation steps and conditions.

Enzyme	Description/source	Substrates	Incubation conditions
1. Pronase-CB	protease from *Streptomyces griseus* (Calbiochem GmbH, Frankfurt)	proteins	sodium-phosphate buffer (0.066 mol/l, pH 7.5, 40 °C)
2. Lipase	triacylglycerollipase from *Candida cylindracea* (Sigma, Munich)	lipids	Tris-HCl buffer (0.2 mol/l; 0.05 mol/l $CaCl_2$, pH 8.0, 37 °C)
3. Cellulase "Onozuka" R-10	cellulase (main activity) from *Trichoderma viride* (Yakult Pharmaceutical Industry Co, Ltd.; Nishinomiya)	cellulose hemicellulose pectin (poly-galacturonic acids)	sodium-acetate buffer (0.17 mol/l, pH 5.0, 40 °C)
4. Amylase Type VI A	α-amylase (main activity) from porcine pancreas (Sigma, Munich)	starch, maltose	potassium phosphate buffer (0.02 mol/l; 0.01 mol/l NaCl, pH 6.9, 40 °C)
5. Cellulysin™ -Cellulase	cellulase (main activity) from *Trichoderma viride* (Calbiochem GmbH, Frankfurt)	cellulose (starch, hemicellulose, proteins)	sodium-acetate buffer 0.1 mol/l, pH 5.0, 40 °C)

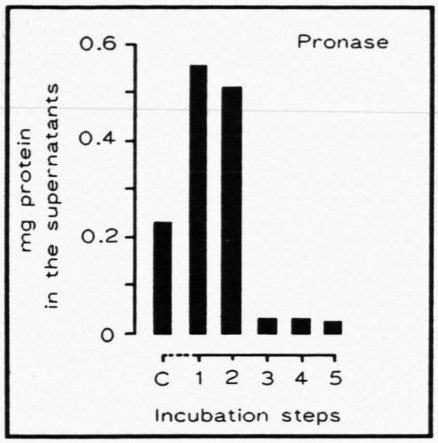
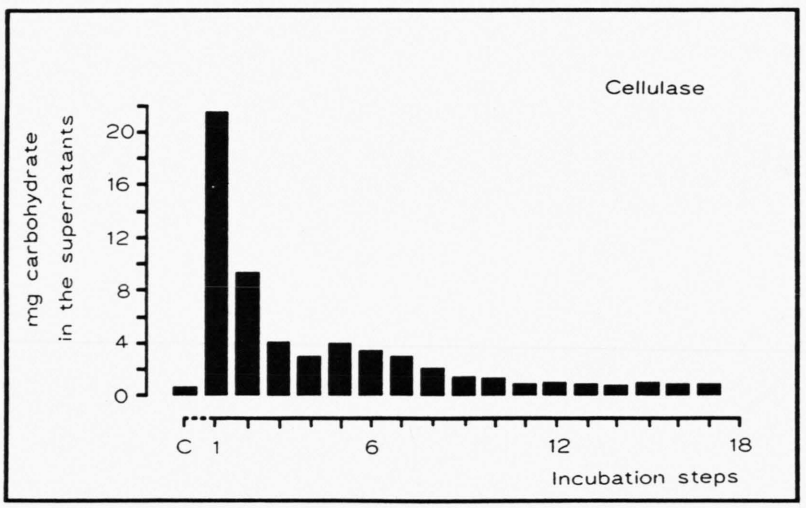
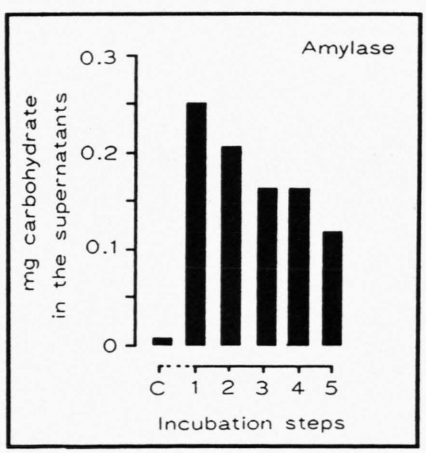
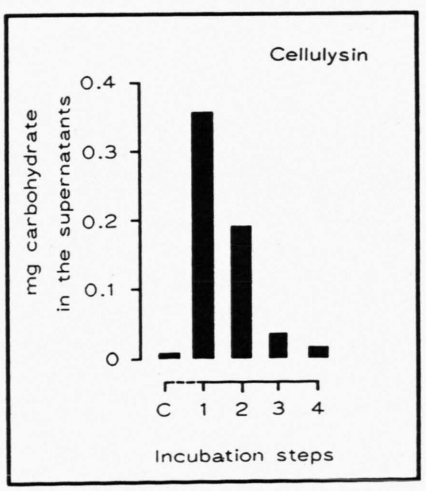


Fig. 2. The amount of hydrolysis products released during incubation of the wall fraction from *Corylus* pollen with hydrolytic enzymes. [For detail see Fig. 1, Tab. I C: control = the supernatants of the washing step before the enzyme treatments. b, c, d: glucose was used as a standard].







MS, indicated that mainly glucose, and arabinose, and some unidentified components appear as hydrolytic products from the wall material. Initially, mainly glucose was produced during the cellulase treatment, later on arabinose. The detection of arabinose is in good agreement with the observation of Bouveng [17] investigating the intine material from *Pinus* pollen.

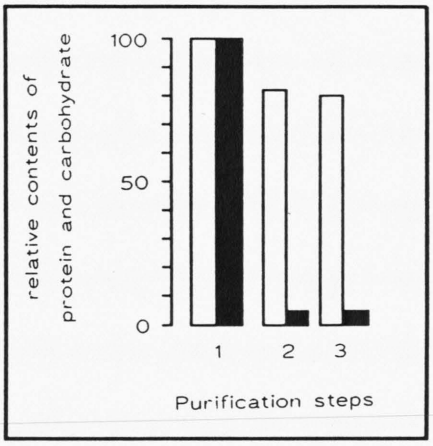


Fig. 3. The course of the purification procedure of "Onozuka" R 10 cellulase. 1: crude enzyme, 2: the enzyme after precipitation with 80% $(NH_4)_2SO_4$, 3: the enzyme after fractionation on a trisacryl column.
□: protein, ■: carbohydrates.

A subsequent treatment of the intine-free pollen walls with solvents, especially with methanol, was necessary to extract non-covalently bound substances from the exine structure. Our interest was focussed especially on the hydroxycinnamic acid amides, diferuloyl- and caffeoylferuloyl spermidine, which are known to be soluble components of the exine [11]. After an extraction period of 143 days no more spermidines could be detected. The results of an elemental analysis of the completely extracted wall material indicated that no more nitrogen containing compounds were present (Table II). At the end of the whole purification procedure intine-free material was obtained, a fact that was confirmed by electron microscopic analyses. This material was designated as sporopollenin fraction (Fig. 1).

Table II. The results of elemental analyses of the pollen wall material at different stages of the purification procedure.

Material	Elementary composition of the pollen wall fractions (%, W/W)	
pollen wall fragments before treatment with enzymes and solvent extraction	C H N	55.61 7.65 1.54
pollen wall fragments after treatment with enzymes and methanol extraction (= 1515 h)	C H N	61.54 8.34 0.72
pollen wall fragments after enzyme treatment and after exhaustive extraction with solvents (= 3428 h)	C H N	61.24 8.38 0.00

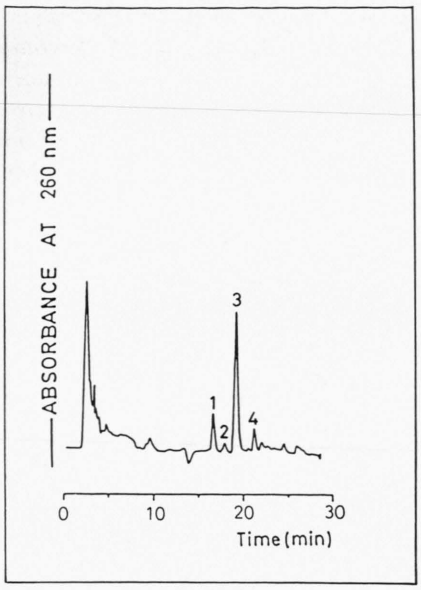
Investigation of the degradation of a highly purified sporopollenin fraction

Potash-fusion

Following degradation of sporopollenin by potash-fusion different compounds were released. In addition to some not yet identified compounds, which appeared without exception at low concentrations, the major decomposition compound was *p*-hydroxybenzoic acid (Fig. 5 and 2).

Nitrobenzene oxidation

Nitrobenzene oxidation degraded sporopollenin with nearly 100% efficiency. The acidified diethylether extracts following nitrobenzene oxidation were analyzed by TLC and HPLC. Each chromatogram indicated that phenolic substances like *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde and vanillin were formed (Fig. 4). In addition to these compounds, some not yet identified compounds, which were to some extent generated by the reaction mixture (NaOH + nitrobenzene), were found. *p*-Coumaric acid appeared in only very small amounts under the appropriate experimental conditions. A comparison of these results with those ob-



tained by Schulze Osthoff and Wiermann [5] demonstrates that the production of *p*-coumaric acid depends strongly on the condition under which nitrobenzene oxidation was carried out. Quantitative analyses indicate, that *p*-hydroxybenzaldehyde is the major compound. The ratio of the individual decomposition products are summarized in Table III.

It is reasonable to assume that NBO treatment of sporopollenin results in a considerable loss and/or chemical modification of the originally occurring compounds. This effect was studied in control experiments in which reference substances such as ferulic acid, *p*-coumaric acid, vanillin, vanillic acid, *p*-hy-

Fig. 4. HPLC diagram obtained with the acidified ether extract following nitrobenzene oxidation of a highly purified sporopollenin fraction from *Corylus* pollen [1: *p*-hydroxybenzoic acid 2: vanillic acid 3: *p*-hydroxybenzaldehyde 4: vanillin].

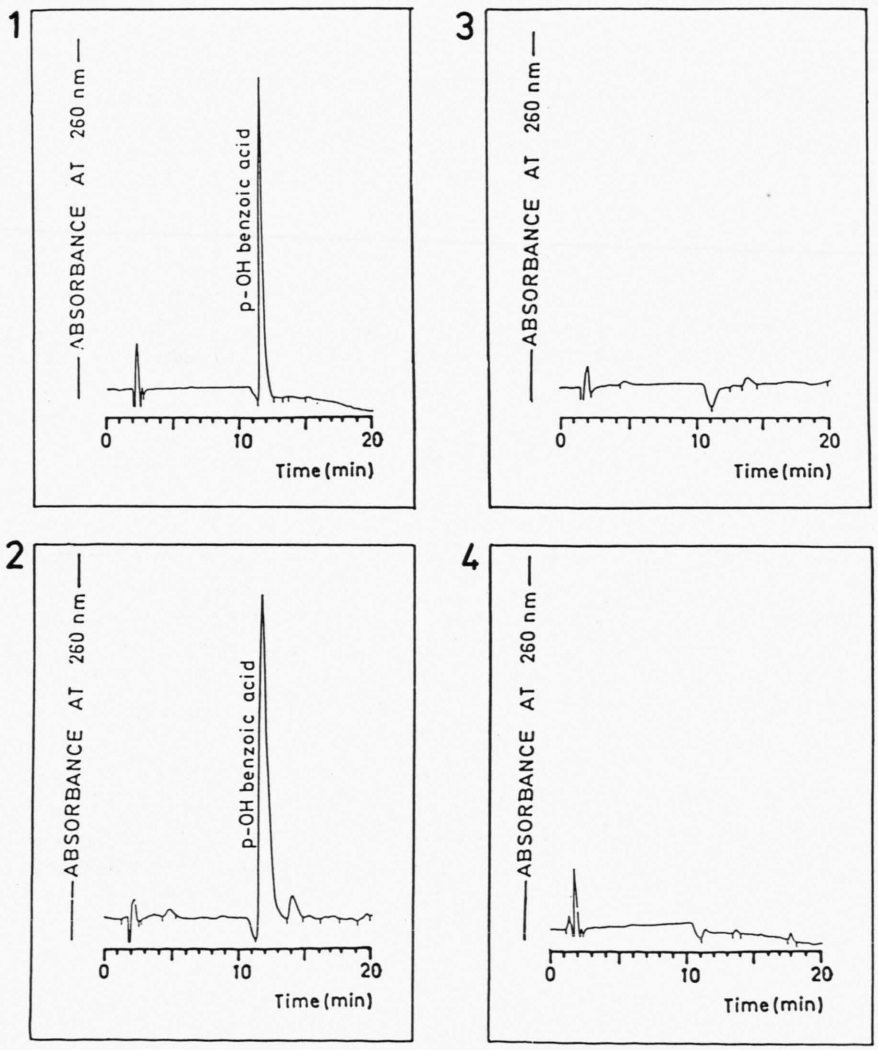


Fig. 5. HPLC diagrams obtained with the acidified ether extract following potash-fusion (1): reference substance, (2): assay with *Corylus* sporopollenin, (3): assay with "synthetic sporopollenin", (4): control assay without natural or "synthetic sporopollenin".

droxybenzaldehyde and *p*-hydroxybenzoic acid were subjected to NBO treatment under identical conditions. The amounts of compounds such as *p*-coumaric acid and ferulic acid changed drastically. At the end of the reaction nearly all of these substances were present as *p*-hydroxybenzaldehyde or vanillin.

Following degradation of sporopollenin by saponification with 6 N KOH at a reaction temperature of 100 °C *p*-coumaric acid was identified as the major product in addition to ferulic acid, which was also present in large amounts. In this case control studies demonstrated that both *p*-coumaric acid and ferulic acid were stable. Following the degradation of sporopollenin using two different methods with the proper controls it appears that, although *p*-hydroxybenzaldehyde seems to be the major component of the phenolic compounds, *p*-coumaric acid is probably the true major component of sporopollenin. It is not possible to quantify the detected components because little is known about the actual structure of sporopollenin and the binding types within it.

Studies with "synthetic sporopollenin"

The appearance of phenolic compounds following the degradation of sporopollenin by potash-fusion was interpreted by Brooks and Shaw [18] to be due to the degradation of carotenoid skeletons under rigorous experimental conditions. We therefore degraded "synthetic sporopollenin" exactly under the same conditions like natural sporopollenin and analyzed the products formed by TLC and HPLC (Fig. 5–7). From our data it cannot be excluded that the same and/or similar phenolic compounds were formed during the degradation of "synthetic sporopollenin". However, identification of the compounds was not possible due to the small amounts. Similar signals detected by HPLC appeared in control experiments, which contained only the reaction mixture but neither natural nor "synthetic sporopolle-

Table III. The amount of phenolics from *Corylus* sporopollenin degradated by NBO.

Extracted substances	µg/mg sporopollenin of *Corylus avellana*
p-hydroxy benzoic acid	1.99
vanillic acid	0.96
p-hydroxy benzaldehyde	12.09
vanillin	3.99
p-coumaric acid	0.84
total	19.87
percentage of the exine material	1.99 %

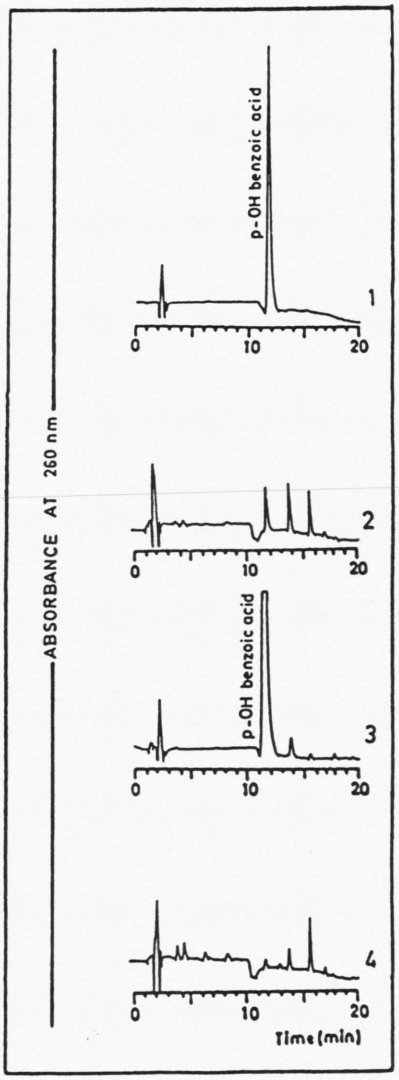


Fig. 6. HPLC diagrams obtained with the eluates from the spots corresponding with *p*-hydroxybenzoic acid after TLC of the acidified ether extract following NBO (TLC-2, 3). 1: reference substance, 2: control assay without natural or "synthetic sporopollenin", 3: assay with *Corylus* sporopollenin, 4: assay with "synthetic sporopollenin". The quantities applied to the HPLC in 3 were only a third part of those in 2 and 4.

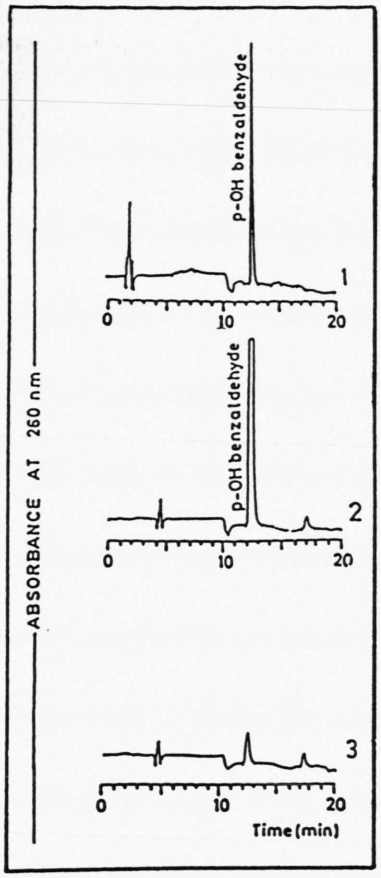


Fig. 7. HPLC diagrams obtained with the eluates from the spots corresponding with *p*-hydroxybenzaldehyde after TLC of the acidified ether extract following NBO (TLC-1). 1: reference substance, 2: assay with *Corylus* sporopollenin, 3: assay with "synthetic sporopollenin". The quantities applied to the HPLC in 2 were only a fifth part of those in 3.

nin". Quantitative comparative analyses of equal assays with natural and "synthetic sporopollenin" indicated that, if phenols are actually present as degradation products following degradation of the "synthetic sporopollenin" by means of potash-fusion or NBO, these substances are present only in extremely small amounts in comparison to the amounts obtained after degradation of *Corylus* sporopollenin (Fig. 5–7).

Discussion

In order to identify the structure of sporopollenin, degradation experiments with subsequent analyses of low molecular weight products were carried out. Sporopollenin was not isolated and purified by the often used rigorous methods such as acetolysis or treatment with phosphoric acid for 10 days [9, 10], but with the help of different hydrolytic enzymes and by exhaustive extraction of non-covalently bound components. By this method an exine material free of contamination with intine components or other non-covalently bound material, designated as sporopollenin fraction was obtained in a sufficient quantity for degradation experiments by potash-fusion and NBO. With the isolation and enrichment of "pollen wing" material from *Pinus* pollen [5] or by separating the exine with 4-methylmorpholine N-oxide monohydrate [19] two additional methods yielding exine material have been described. It is not clear whether the latter method produces enough material for degradation studies.

Nowadays sporopollenin is often thought to be a biopolymer, derived biogenetically from carotenoids or carotenoid esters. As shown previously [5] and in this study different quantities of phenolic compounds were released following potash-fusion and NBO. The main compound after potash-fusion of *Corylus* sporopollenin could be identified as *p*-hydroxybenzoic acid while *p*-hydroxybenzaldehyde was the main product after NBO. At present it is not known whether, and if so to which extent, *m*-hydroxybenzaldehyde occurs in addition to *p*-hydroxybenzaldehyde. This question is still under study. The degradation of sporopollenin by means of saponification and NBO and of reference substances demonstrates, that the true major component is *p*-coumaric acid and not *p*-hydroxybenzaldehyde. According to the interpretation by Brooks and Shaw [1, 18] degradation products such as *p*- and *m*-hydroxybenzoic acid should be generated from carotenoid skeletons as a result of the forcing experimental conditions following potash-fusion. The results clearly demonstrate that phenolic compounds, if they indeed resulted from the degradation of "synthetic sporopollenin", are generated only in extremely small quantities in comparison to those produced by degradation of *Corylus* sporopollenin. We conclude, that phenolic components are integral constituents of sporopollenin. The true portion of phenols is not known, because 1) the experimental conditions for the degradation of the sporopollenin are not optimized and, 2) it cannot be estimated to which extent phenolic structures of the sporopollenin are degraded and lost under the

experimental conditions chosen. The experimental data and estimations by Allan and Mattila [20] show that a marked loss of aromatic structures occurs during potash-fusion. Our conclusions, that phenolic compounds are elements in the structure of sporopollenin is strongly supported by tracer experiments which resulted in a good incorporation of [^{14}C]phenylalanine into the sporopollenin fraction [4; Rittscher and Wiermann in preparation]. After subsequent degradation of the ^{14}C-labelled sporopollenin by means of potash-fusion the majority of the radioactivity was found within *p*-hydroxybenzoic acid (Rittscher and Wiermann in preparation).

Acknowledgements

The work was supported by a grant of the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We are indebted to Dr. Geisert, Institut für Physiologische Chemie, Mainz, for generous gift of samples of "synthetic sporopollenin" and to R. and Dr. J. Holtum (Münster) for revising the English.

[1] J. Brooks and G. Shaw, Pollen Development and Physiology (J. Heslop-Harrison, ed.), pp. 99–114, Butterworths, London 1971.
[2] J. Brooks and G. Shaw, Trans. Bose Res. Inst. (Calcutta) **40,** 19–38 (1977).
[3] A. K. Prahl, H. Springstubbe, K. Grumbach, and R. Wiermann, Z. Naturforsch. **40c,** 621–626 (1985).
[4] A. K. Prahl, M. Rittscher, and R. Wiermann, in: Biotechnology and ecology of pollen (D. L. Mulcahy, G. B. Mulcahy, and E. Ottaviano, eds.), pp. 313–318, Springer Verlag, New York, Berlin, Heidelberg, Tokyo 1986.
[5] K. Schulze Osthoff and R. Wiermann, J. Plant Physiol. **131,** 5–15 (1987).
[6] G. Shaw, in: Sporopollenin (J. Brooks, P. R. Grant, M. Muir, P. van Gijzel, and G. Shaw, eds.), pp. 305–348, Academic Press, London, New York 1971.
[7] D. Southworth, J. Histochem. Cytochem. **21,** 73–80 (1973).
[8] D. Southworth, Amer. J. Bot. **61,** 36–44 (1974).
[9] G. Erdtman, Sven. bot. Tidskr. **54,** 561–564 (1960).
[10] D. Green, PhD Thesis, University Bradford 1973.
[11] S. Gubatz, S. Herminghaus, B. Meurer, D. Strack, and R. Wiermann, Pollen et Spores **XXVIII,** 347–354 (1986).
[12] E. Stahl, Dünnschichtchromatographie. Ein Laboratoriumshandbuch, 2. Auflage, Springer Verlag, Berlin, Heidelberg, New York 1967.
[13] M. Somogyi, J. Biol. Chem. **195,** 19–23 (1951).
[14] D. Aminoff, W. Binkli, R. Schaffer, and R. W. Mowry, in: The Carbohydrates, Chemistry and Biochemistry (W. Pigman and D. Horten, eds.), **Vol. II B,** 739–807, Academic Press, New York, San Franzisco, London 1970.
[15] M. M. Bradford, Anal. Biochem. **72,** 248–254 (1976).
[16] S. M. Read and D. H. Northcote, Anal. Biochem. **116,** 53–64 (1981).
[17] H. O. Bouveng, Acta Chem. Scand. **19,** 953–963 (1965).
[18] J. Brooks and G. Shaw, Nature **219,** 532–533 (1968).
[19] F. A. Loewus, B. G. Baldi, V. R. Franceschi, L. D. Meinert, and J. J. McCollum, Plant Physiol. **78,** 652–654 (1985).
[20] G. G. Allan and T. Mattila, in: Lignins – occurrence, formation, structure and reactions (K. V. Sarkanen and C. H. Ludwig, eds.), pp. 575–596, Wiley-Interscience, New York, London, Sidney, Toronto 1971.