Continuous Decomposition of Sporopollenin from Pollen of *Typha angustifolia* L. by Acidic Methanolysis

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Sporopollenin from the pollen of *Typha angustifolia* L. was exposed to a series of 36 subsequent acidic methanolysis procedures. The remaining decomposition products were investigated using several spectroscopic methods including Fourier transform infrared spectroscopy (FT-IR), solid state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-CPMAS-NMR) and X-ray photoelectron spectrometry (XPS). Substantial weight losses of the sporopollenin material occur after each acidic methanolysis step, while FT-IR and ^{13}C-CPMAS-NMR spectra display no noticeable differences after 12, 24 and 36 steps. These findings are interpreted as a hint that the sporopollenin polymer has a uniform composition, i.e. relatively small monomer moieties of similar primary structure are present. Moreover, the weight losses account for the presence of substantial amounts of ether linkages in the sporopollenin polymer.

Introduction

The biopolymer sporopollenin is the main component of the outer walls of spores and pollen. This substance is extremely resistant to non-oxidative chemical and biological degradation procedures. Because of this resistance, little is known about the definite chemical structure and the biochemical pathways involved in its structure. At present, it is assumed that sporopollenin is a polymer consisting mainly of unbranched aliphatics with a variable amount of aromatics (for review see (Wiermann *et al.*, 2001) and literature cited therein). In the past, spectroscopic techniques played an important role in the elucidation of sporopollenin structure and chemical composition. In this paper, these techniques including Fourier transform infrared spectroscopy (FT-IR), solid state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-CPMAS-NMR) and X-ray photoelectron spectrometry (XPS) were used to elucidate structural changes arising from a stepwise decomposition of sporopollenin from the pollen of *Typha angustifolia* by a long-time treatment with acidic methanolysis. The analyses focussed on the question whether the sporopollenin is of a largely heterogeneous or homogeneous composition.

Experimental

For all investigations sporopollenin of *Typha angustifolia* L. was used. Isolation and purification were performed as described earlier (Ahlers *et al.*, 2000).

Acidic methanolysis

Sporopollenin samples of series A (0.926 g) and series B (1.005 g) were heated under reflux in 100 ml methanolic HCl (0.1 N) at 70 °C for 24 h each. (During reflux, series A samples were stirred with a Teflon stirring bar, whereas series B samples were not stirred at all.) After 24 h of reflux, the samples were centrifuged for 10 min, the liquid phase was decanted and neutralised with $BaCO_3$. Afterwards, residual $BaCO_3$ was removed by centrifugation. The liquid phases were stored in a refrigerator.

The solid phase obtained after the first centrifugation was then added to 100 ml of fresh metha-

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nolic HCl (0.1 N) and refluxed at 70 °C for another 24 h. This procedure was repeated four times. The solid phase obtained after the fifth methanolysis treatment was then washed with distilled water, until the washing water had a pH of 7. After dissolution of the solid phase in a small amount of water and freeze drying of the solution the mass was determined gravimetrically. CHN analysis, FT-IR, XPS and ^{13}C-CPMAS-NMR were performed after the 12th, 24th and 36th methanolysis step.

CHN analysis

The CHN analyses were carried out with a Haereus CHN Rapid analyser. Values are given in Table II and the stoichiometric factors of the sum formula $CH_xO_yN_z$ are listed in Table III.

XPS analysis

Investigations by XPS on electrically non-conductive samples like sporopollenin involve the risk that the samples charge to a positive voltage due to the emission of photoelectrons generated by X-ray excitation. A preparation method was developed that avoids or reduces charging as much as possible and is described elsewhere (Ahlers *et al.*, 2000).

Photoelectron spectra were recorded on an X-ray photoelectron spectrometer AXIS-HS (Kratos, Manchester, UK) using the following conditions: *excitation*: non-monochromatic Al Kα radiation was used; *analysis*: take-off angle for the electrons 0 ° with respect to the surface normal of the sample holder, fixed analyser transmission (FAT) mode, pass energy of the concentric hemispherical analyser (CHA) 20 eV, data acquisition with 0.2 eV per step. The residual gas pressure was 4×10^{-7} Pa. A prevention of charging of the samples could not completely be reached.

^{13}C-CPMAS-NMR

Solid state NMR spectra were measured with a BRUKER Avance 500 WB at a frequency of 125,772 MHz using a 4 mm MAS probehead. The rotation frequency at the magic angle was 12.5 kHz. Between 682–12,288 scans were accumulated, the frequency range was 50 kHz for all measurements. The acquisition time was 20.5 ms. The relaxation delay time was 5 s throughout. The standard cross polarisation pulse program with a contact time of 2 ms was used for all measurements.

FT-IR

IR spectra were obtained with a BRUKER IFS28 spectrometer in the Fourier Transform mode. Samples were measured in KBr. The resolution was 4 cm^{-1}. Spectra were extracted from the wavenumber region from 4000–500 cm^{-1}.

Results and Discussion

For the characterisation of both the purified non-treated and the modified sporopollenin, several spectroscopic methods including Fourier transform infrared spectroscopy (FT-IR), solid state ^{13}C nuclear magnetic resonance spectroscopy (^{13}C-CPMAS-NMR) and X-ray photoelectron spectrometry (XPS) were applied. FT-IR spectroscopy gives information on functional groups that can be deduced from the occurrence of characteristic group frequencies. In the past, solid state NMR was the most widely applied spectroscopic technique in sporopollenin research. Each distinct carbon chemical environment gives rise to a peak at a characteristic chemical shift. XPS is a surface analytical technique that can supply information on the kind of elements in the sample, their binding states and their chemical composition. This technique has only rarely (Ahlers *et al.*, 2000; Kawase and Takahashi, 1995) been applied to the structure elucidation of sporopollenin.

^{13}C-CPMAS-NMR spectra of series A and B show no noticeable differences, nor do the spectra after 12, 24 and 36 procedures of acidic methanolysis. For this reason, only the ^{13}C-CPMAS-NMR spectra of series B (Fig. 1) as well as the FT-IR spectra of series B (Fig. 2) are displayed. As substantial weight losses of the sporopollenin material occur after each acidic methanolysis step, the similarity of the spectra after 12, 24 and 36 procedures of methanolysis may be interpreted as a hint that the sporopollenin polymer has a uniform composition, i.e. relatively small monomer moieties of similar primary structure are present. Moreover, the weight losses indicate that substantial amounts of the sporopollenin polymer are linked via ether bridges. These findings are in accordance with the

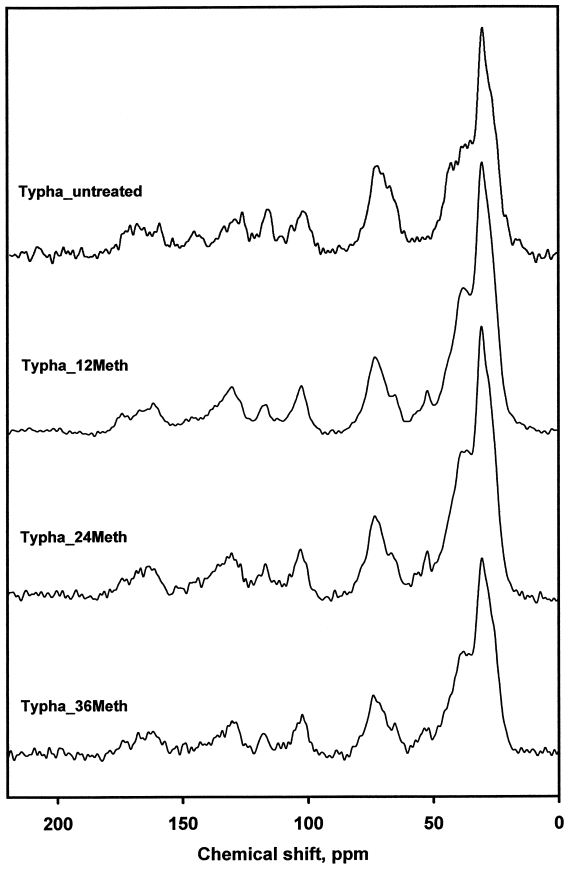

Fig. 1. ^{13}C-CPMAS-NMR spectra of sporopollenin from the pollen of *Typha angustifolia* L. Samples: Untreated sporopollenin (*Typha*_untreated), sporopollenin after 12 (*Typha*_12Meth), 24 (*Typha*_24Meth) resp. 36 (*Typha*_36Meth) steps of acidic methanolysis treatment (series B samples, for details see Experimental part).

Table I. Assignments of IR bands of the sporopollenin samples from the pollen of *Typha angustifolia* L. after acidic methanolysis.

Wavenumber [cm^{-1}]	Assignment
3425	OH-stretching vibrations
2925, 2850	CH-stretching vibrations of saturated carbons
1625	N-H bending vibration
1715	C = O-stretching vibration of aryl esters
840	CH-out of plane vibration of aromatics

Chemical shifts δ and assignments for ^{13}C-NMR signals of the sporopollenin samples from the pollen of *Typha angustifolia* L. after acidic methanolysis.

Chemical shift δ [ppm]	Assignment
30	$(CH_2)_n$ (n > 10) of alkyl chains
37	CH_2 connected to ester carboxyl
72	CH-O-functions from ethers, esters or alcohols
128, 132	aromatic carbons of benzoic acid or ester
160, 165	ester carboxyl

results of other authors (Dominguez *et al.*, 1999), who find that sporopollenin is a complex polyether with a high degree of crosslinking, presumably derived from the polymerisation of polyunsaturated fatty acids.

All IR spectra of sporopollenin obtained after different modification procedures (Ahlers *et al.*, 2000) or from different plant taxa (Wiermann *et al.*, 2001) show a sharp peak at 1510 cm^{-1}, that could not yet be assigned to a functional group. The FT-IR spectra (Fig. 2) of all samples show bands at 2925 and 2850 cm^{-1} arising from alkyl chains (Table I), this assignment being supported by the occurrence of the strong ^{13}C-NMR signal at 30 ppm (Table I) characteristic of chains with at least ten CH_2 groups. These results are in accordance with the work of Blokker (Blokker *et al.*, 1999), who find linear chain $C_{22}-C_{30}$ alcohols and carboxylic acids as constituents of a sporopollenin-like wall material isolated from the green alga *Chlamydomonas monoica.* Biochemical studies using thiocarbamate herbicide inhibitors of the chain-elongating enzyme systems of lipid metabolism indicate that the lipid metabolism and the chain-elongating systems are involved in the biosynthesis of sporopollenin (Wilmesmeier and Wiermann, 1995). Furthermore, the importance of the aliphatic metabolism in sporopollenin biosynthesis could be confirmed by tracer experiments (Meuter-Gerhards *et al.*, 1999). The importance of aliphatic structures is also stressed by Kawase and Takahashi (Kawase and Takahashi, 1995), who conclude that the main structure of sporopollenin is a simple aliphatic polymer containing aromatic or conjugated side chains. Moreover, CHN analysis data (Table II), corresponding to a sum formula in the range $CH_{1.49}O_{0.27}$ to $CH_{1.57}O_{0.28}$, confirm that approximately half of the sporopollenin carbons are bonded as CH and the other half as CH_2, CH_3 carbons (δ_C = 0 - 20 ppm) as well as quater-

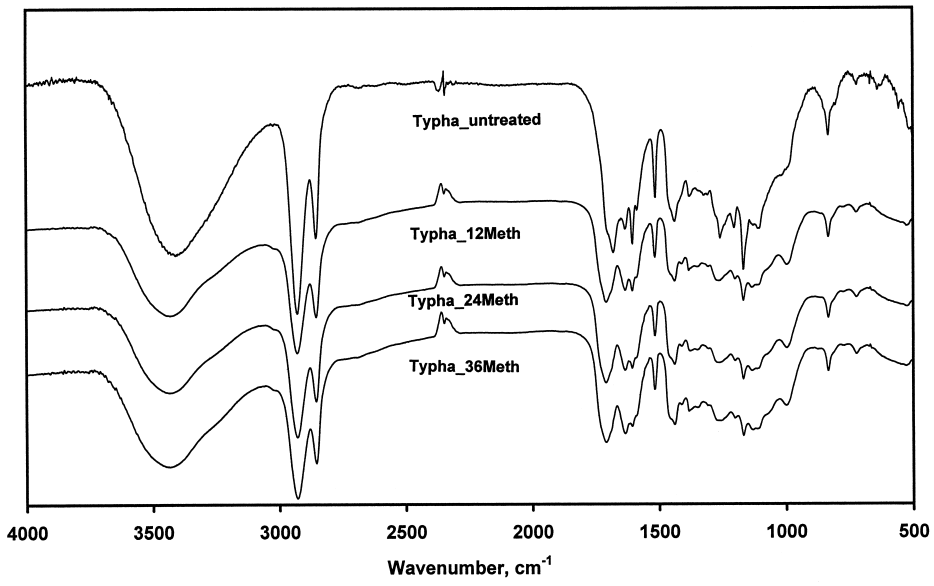

Fig. 2. FT-IR spectra of sporopollenin from the pollen of *Typha angustifolia* L. Samples: Untreated sporopollenin (*Typha*_untreated), sporopollenin after 12 (*Typha*_12Meth), 24 (*Typha*_24Meth) resp. 36 (*Typha*_36Meth) steps of acidic methanolysis treatment (series B samples, for details see Experimental part).

nary carbons (δ_C = 160 ppm) are low in intensity in the ^{13}C-CPMAS-NMR spectra (see Fig. 1).

As the nitrogen content of the samples is, according to the XPS investigations, considerably lower than the oxygen content (Fig. 3), the NMR signal at 165 ppm (Table I) is probably due to a carboxyl function, i.e. an acid or ester function, and not due to amide. Moreover, the position of the carboxyl band in the FT-IR spectra at 1715 cm^{-1} is characteristic of an aryl ester (Table I), which is in accordance with the observations of Ahlers (Ahlers *et al.*, 2000), who found an α,β-unsaturated carbonyl group in the sporopollenin of *Typha angustifolia L.* The appearance of aromatic carbon signals in the ^{13}C-CPMAS-NMR spectrum in a rather narrow signal range around 130 ppm (Table I) supports the occurrence of benzoic acid moieties (Hesse *et al.*, 1995). NMR signals around 72 ppm (Table I) are characteristic of CHO-carbons originating from hydroxyl-, ester- or ether linkages.

Table II. Elemental contents (wt-%) determined by CHN analysis of the sporopollenin samples from the pollen of *Typha angustifolia* L. after acidic methanolysis.

Sample	Elemental content			
	C	H	N	O
A12	66.89	8.65	0.00	24.46
A24	66.78	8.30	0.00	24.92
A36	67.02	8.76	0.00	24.22
B12	66.66	8.68	0.00	24.66
B24	67.07	8.79	0.00	24.14
B36	67.25	8.72	0.00	24.03

The elemental contents of C, H and N as determined from CHN analysis are given in Table II. Whether nitrogen must be considered as an integral component of the sporopollenin structure, cannot be convincingly shown. Generally, sporopollenin is regarded as a polymer containing no nitrogen (Zetzsche and Vicari, 1931; Shaw, 1971). Sporadic reports pointing to a presence of nitrogen in sporopollenin might be due to a contamination of the material during preparation (Jungfermann *et al.*, 1997). The content of O was obtained by supplementing the total content to 100 wt-%. The uncertainty of the content determination amounts to about 0.05 wt-%. The elemental contents of C, O and N obtained from XPS spectra are listed in Table III together with the content of H which has been calculated using the ratios of the atomic contents of C to H given in Table II. The error of the content determination with the quantitative XPS analysis was estimated by repetitive measurements of different spots on various samples and includes the sampling error, preparation inhomogeneities and the instrumental and evaluation error as well. It can be described best by Δc =

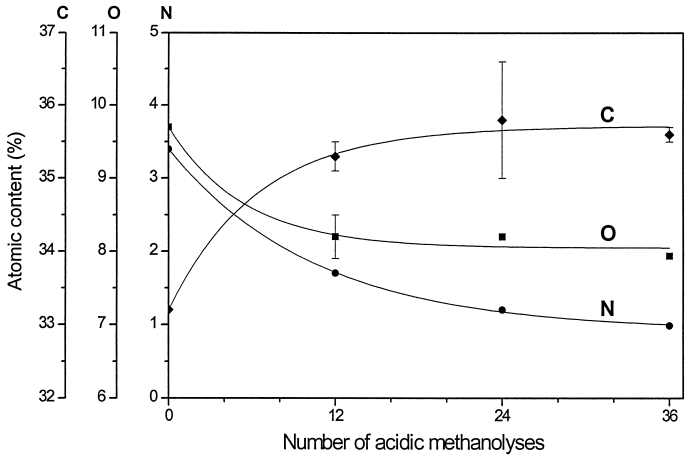

Fig. 3. Atomic contents, determined by XPS, of carbon, oxygen and nitrogen of sporopollenin from the pollen of *Typha angustifolia* L. after 12 (a), 24 (b) resp. 36 (c) steps of acidic methanolysis treatment (series A samples, for details see Experimental part).

$(1 \times 10^{-5} + 2 \times 10^{-3}\ c^2)^{0.5}$, where c is the measured content. As can be seen from Table III no significant differences exist between the series A and B so that the values of these two series have been grouped together and only the corresponding mean values will be considered in the following. The atomic contents of C, O and N taken from Table III are displayed in Fig. 3. The nitrogen content decreases with increasing number of acidic methanolyses, the carbon content increases, while the oxygen content remains fairly constant after the first methanolyses. The most decisive changes, however, occur during the first steps of acidic methanolysis resulting in a decrease as shown in Fig. 3.

The stoichiometric factors for H, O and N calculated from CHN analysis as well as from XPS analysis are listed in Table IV. It will be noticed that the sum of the stoichiometric factors y and z, measured for O and N by XPS, are nearly that stoichiometric factor y, obtained by CHN analysis.

For a more detailed analysis the measured photoelectron spectra have to be fitted by a set of lines positioned at different binding energies which give information about the binding states of the elements (Ahlers *et al.*, 2000). Due to the uncertainty in fixing the binding energies by this deconvolution procedure the error of the content determination is enlarged by a factor of 3 to 4. The binding energies together with the corresponding, most probable functional groups are listed in Table V. The portion of the amino groups (CNH_x) as well

Table III. Normalised elemental contents (at-%) determined by XPS measurements of the sporopollenin samples of series A and B after 12, 24 and 36 procedures of acidic methanolysis (samples denoted A12–36 and B12–36, respectively).

Sample	Contents for			
	H[a]	C	O	N
Native	53.7	33.2	9.7	3.4
A12	54.9	35.4	8.0	1.7
B12	54.9	35.1	8.4	1.7
Mean	54.9 ± 0.0	35.3 ± 0.2	8.2 ± 0.3	1.7 ± 0.0
A24	54.2	36.3	8.2	1.3
B24	55.4	35.2	8.1	1.2
Mean	54.8 ± 0.9	35.8 ± 0.8	8.2 ± 0.0	1.2 ± 0.0
A36	55.6	35.5	7.9	0.99
B36	55.5	35.6	7.9	0.98
Mean	55.5 ± 0.1	35.6 ± 0.1	7.94 ± 0.0	0.99 ± 0.01

[a] H was calculated using the ratio of the atomic contents of H to C determined by CHN analysis.

Table IV. Stoichiometric factors x, y, z of the sum formula $CH_xO_yN_z$ determined by CHN and XPS analysis of the sporopollenin samples of series A and B after acidic methanolysis.

Sample	CHN		XPS	
	x	Y	y	z
A12	1.55	0.27	0.23	0.05
A24	1.49	0.28	0.23	0.04
A36	1.57	0.27	0.22	0.03
B12	1.56	0.28	0.24	0.05
B24	1.57	0.27	0.23	0.03
B36	1.56	0.27	0.22	0.03

Table V. Normalised contents (at-%) for C, O and N in different functional groups after methanolysis of sporopollenin.

Element	Binding energy (eV)	Functional groups	Number of acidic methanolyses		
			12	24	36
C	285.0	CC CH	57.6	59.8	61.7
C	286.6	COR CNH_x	12.6	12.2	11.7
O	532.8	COR	8.9	9.5	9.5
N	400.3	CNH_x	3.8	2.7	2.2
C	288.0	C = O	4.7 ± 0.5	4.1 ± 1.3	3.5 ± 0.7
O	531.6				
C	289.0	COOR	3.7 ± 0.4	3.6 ± 0.3	3.6 ± 0.5
O	534.1				

as of the carbonyl groups (C = O) decreases with increasing number of acidic methanolyses. These decreases are accompanied with an increase of CC and/or CH bonds, while other functional groups like alkoxyl ones (COR or COH, resp.) and ester and/or carboxyl groups (COOR and/or COOH, resp.) remain constant. This trend is in accordance with the results of IR spectroscopy, that show a relative decrease of the intensity of the band of the NH-stretching vibration relative to the intensity of the carboxyl band with increasing number of methanolyses (Fig. 2). The behaviour of the carbonyl and carboxyl groups can be drawn from the C signal and the O signal as well (see the last two lines in Table V). The relatively high binding energy of 286.6 eV for COR points to the presence of ester or epoxy groups and less hydroxyl groups, also in accordance with the results of IR spectroscopy.

Furthermore, sulfur can clearly be indicated at all samples in two different binding states, first as sulfide (ca 0.3 at-%) and as sulfone and/or sulfonate (ca 0.1 at-%). The sulfur content of the samples still remains to be explained; impurities of the samples cannot be excluded.

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