A geochemical study of lacustrine sediments: towards palaeo-climatic reconstructions of high Andean biomes in Colombia

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CHAPTER 5

*p*-Coumaric acid-based sporopollenin of *Isoëtes killipii* megaspores.

The megaspores of *Isoëtes killipii* C. Morton, contain a highly resistant bio-polymeric material often referred to as sporopollenin. Extraction and hydrolysis of the dried megaspores yielded a residue of high chemical purity, which was used for chemical analysis using a series of complementary techniques: DT-MS, pyrolysis-GC/MS, TMAH/pyrolysis-GC/MS and FTIR. The data revealed that the resistant biopolymer in the walls of the megaspores was predominantly constructed from *p*-coumaric acid building blocks closely resembling lignin. Additional evidence was obtained by analysis of the ester-bound lipids removed upon basic hydrolysis of the megaspore walls. Comparison of these results with those obtained from a synthetic *p*-coumaric acid-based dehydrogenation polymer (DHP) indicates that the natural and model (DHP) polymers closely match. Although this is not the first time that *p*-coumaric acid is shown to be a structural unit of sporopollenin, this is the first example of sporopollenin that seems to be for a major part made of *p*-coumaric acid. Our results show that the combination of different analytical techniques is of great use in structure elucidation of the wide variety of biopolymers that fall under the term sporopollenin.
5.1. Introduction

The structure of sporopollenin has been debated for a long period of time (e.g. Brooks et al. 1971; Kawase et al. 1995; Dominguez et al. 1999 and references therein). A wide variety of materials have been analysed over the years and probably even a wider variety of polymeric structures have been proposed. Due to the improvement of different analytical techniques and new insights in the field of plant biopolymers (Wiermann and Gubatz 1992), it is becoming increasingly clear that sporopollenin is probably an umbrella-term for a wide variety of highly resistant biopolymers. Largeau and de Leeuw (1993) published the first critical review on sporopollenin and propose to restrict this term to only the macromolecular component of the outer walls of pollen and spores of vascular plants. These polymers have the tendency to preserve in ancient sediments and soils, because of their resistance to biodegradation and therefore they are also interesting from a geochemical point of view. The physiological function of the sporopollenin is likely to be related to its highly resistant nature and could serve to protect the endospore to a variety of environmental and chemical hazards.

Plants of the genus Isoëtes (Isoetaceae) are omnipresent and are commonly found in aquatic or semi-aquatic environments (Mauseth 1998). They produce large quantities of male micro and female megaspores which easily end up in surface sediments, where they need protection against many degradation processes in order to survive. The sporangia with the megaspores often end up in the sediment itself, so that the macrospores are sometimes present in massive numbers in sediments deposited at times when Isoëtes was abundant. Quaternary high Andean lacustrine sediments often contain these fossil Isoëtes spores and are sometimes even composed of such a high amount of fossil megaspores that it forms a clearly visible deposit (Cleef 1981). In Quaternary palynological studies of Andean lacustrine sediments, Isoëtes spores are used as indicators of variations in lake levels (Hooghiemstra 1984). However it is unknown whether Isoëtes spores always survive the sedimentation process and subsequent diagenesis with intact morphology. Because of their large dimension (our spores measured approximately 0.7 mm in diameter) the megaspores are highly susceptible to mechanical stress in the sediments, which may change or even destroy the morphology of these spores. When no visible evidence exists for the presence of these spores, their resistant sporopollenin might however still be preserved.

The ultra structure and the location of the sporopollenin layer of Isoëtes megaspores have been described by Taylor (1993), as a layer of an electron translucent material interwoven with the siliceous outer layer and polysaccharide inner wall. The chemical nature of the electron translucent sporopollenin layer remained undefined. The chemical composition of sporopollenin from fossil and extant megaspores of Azolla and Salvinia has been reported by van Bergen (1993 and 1995) and a dominating p-vinyl phenol component in the pyrolysates was attributed to the pyrolysis product of p-coumaric acid. A similar observation was made by Wehling et al. (1989) who reported the dominant presence of p-vinyl phenol amongst the direct temperature-
resolved pyrolysis products of purified *Pinus* sporopollenin. They also ascribed this pyrolysis product to a *p*-coumaric acid based biopolymer.

In this paper, we report the chemical structure of the resistant biopolymer present in the walls of *Isoëtes* megaspores in order to facilitate a chemical investigation of the presence of megaspore remains in ancient sediments. Furthermore, the elucidation of the chemical structure of this sporopollenin contributes to the knowledge of these resistant plant materials. We present the GC/MS, Py-GC/MS, DT-MS and on-line methylation with TMAH Py-GC/MS data. We propose that the sporopollenin from the megaspores of the aquatic *Isoëtes killipii* consists of a major part of a *p*-coumaric acid based polymer. The large dimensions of the megaspores enabled purification of the sporopollenin to a high degree of purity that has not been accomplished before.

5.2. Materials and methods

5.2.1. Plant material.

Submerged plants of *Isoëtes killipii* were sampled from a lake at 3090 m altitude in the Paramo de Mirador in the Central Cordillera of Colombia (near Medellin) in July 1997.

5.2.2. Megaspore treatment.

100 mg of megaspores were hand picked from the sporangia, washed subsequently in 5 ml water, 5 ml methanol, 5 ml dichloromethane and dried, leaving 60 mg of dry material. The spores (ca. 1 mm in diameter) were crushed using a small mortar and the fragments were washed with water and then transferred to 5 ml methanol and ultrasonically pulverised. The methanol was discarded after centrifuging. This extraction was repeated 2 times and afterwards repeated using subsequently 3 times a 5 ml methanol/dichloromethane (1:1) mixture and 3 times 5 ml dichloromethane. The residue was refluxed in 5 ml trifluoro acetic acid (2 M) for 2 h and subsequently washed with water until the aqueous phase turned neutral and washed with 5 ml methanol (3x), dried and transferred into 5 ml sulphuric acid (12 M) and allowed to stir for 2 h. The residue was subsequently washed with water, methanol and dichloromethane and dried. Then the residue was refluxed in 5 ml sulphuric acid (2 M), after 1 h the residue was rinsed with water until the aqueous phase turned neutral and washed with 5 ml of methanol (3x), leaving 50 mg of material.

Finally, the residue was saponified for 2 h in a refluxing methanolic solution of KOH (2M, 4% H$_2$O). Then the residue was extracted with 5 ml methanol (2x), 5 ml methanol/dichloromethane (1:1, 2x), 5ml dichloromethane (2x), leaving 30 mg of final residue, which was used for the sporopollenin analyses. The combined solvent fractions obtained after saponification were transferred to a separatory funnel and 20 ml of water was added and adjusted to pH 3 using HCl (2 M). The aqueous phase was extracted with 10 ml of dichloromethane (2x). The combined organic layers were dried over Na$_2$SO$_4$ and the solvents were removed *in vacuo*. The compounds were derivatised with diazomethane and BSTFA.
5.2.3. Dehydrogenation Polymer (DHP) synthesis.

400 mg of p-coumaric acid were dissolved in 10 ml of acetone and transferred to 100 ml acetic acid buffer solution (pH 5.7, 0.2 M), containing 0.3 % H$_2$O$_2$. An aliquot of 5 mg of horseradish peroxidase (Sigma, type II) was stepwise added. After 24 h yellow DHP aggregations were removed from the reaction medium. The aggregates were washed with demineralised water and ethanol to remove free p-coumaric acid. (Yield 5 mg.)

5.2.4. Gas chromatography-mass spectrometry (GC/MS).

GC/MS was performed using a Hewlett Packard 5890 Series II gas chromatograph coupled to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range m/z of 800-50 and a cycle time of 1.6 s. The GC was equipped with a fused silica capillary column (25 m x 0.32 mm) coated with CP-Sil 5 (film thickness 0.12 μm). Carrier gas was Helium. On column injection was used. All identified compounds are given in table 5.1 at the end of this chapter.

5.2.5. Curie-point pyrolysis-gas chromatography mass-spectrometry (Py-GC/MS).

Py-GC/MS was performed using a Hewlett Packard 5890 Series II gas chromatograph with a FOM-3LX pyrolysis unit, interfaced to a VG Autospec Ultima mass spectrometer operating at 70 eV, resolution 1000 with a mass range m/z of 800-50 and a cycle time of 1.6 s. A small amount of the sample was applied to a ferromagnetic wire with a Curie-point temperature of 610°. The GC was equipped with a fused silica capillary column (25 m x 0.32 mm) coated with CP-Sil 5 (film thickness 0.40 μm). Helium was used as a carrier gas.

5.2.6. Direct Temperature-resolved mass-spectrometry (DT/MS).

DT/MS experiments were performed with a JEOL DX-303 double focusing mass spectrometer (E/B mode) operating at 16 eV, resolution 1000 with a mass range m/z of 1000-20 and with a cycle time of 1 s. An aliquot of sporopollenin suspension in water was applied to a Pt/Rh wire (0.1 mm diameter, 10% Rh) and dried in vacuo. After insertion of the probe into the ion source, the wire was heated resistively at a rate of 16 °C/sec to a final temperature of 800 °C.

5.2.7. Fourier transform infrared spectroscopy (FT-IR).

Fourier transform infrared spectroscopy was performed on a Bruker IFS28, scanning over a frequency range of 400 to 4000 cm$^{-1}$, with 32 scans and a resolution of 2 cm$^{-1}$, using KBr tablets containing 2 mg of dry sporopollenin.
Figure 5.1: Gas chromatograms of the Curie temperature (610°) flash pyrolysates of, A: purified sporopollenin, B: of DHP and C: of pure p-coumaric acid.
5.3. Results and discussion

The structural analysis of sporopollenin or sporopollenin-like materials is strongly hampered by their chemical resistance. It is often impossible to remove structural elements from these biopolymers by means of hydrolysis. In contrast, flash pyrolysis techniques can be regarded as an excellent alternative tool to obtain structural information on polymeric structures by thermally cleaving the biopolymer into fragments amenable to gas chromatography/mass spectrometry (py-GC/MS) (Larter and Horsfield 1993), or direct thermally resolved mass spectrometry (DT/MS).

Our sporopollenin has been isolated and purified using simple but efficient steps. A crucial point in every sample cleanup is to remove as much of the unwanted material as possible, in our case the components of endospore and all components loosely associated with the exospore. Therefore we started off with a mechanical treatment. The relatively large spores are easily crushed and hence the internal spore components are removed easily by extraction. This reduces the possibility of mixing the sporopollenin signal with the chemical component of the spore’s interior. Next the spore walls are broken down by acid hydrolysis (starting mild and ending with concentrated acid) and basic saponification. The material that is left after this treatment is a non hydrolysable saponifiable biopolymer. Following the definition of Largeau and de Leeuw (1993) this is sporopollenin.

5.3.1. Py-GC/MS

Py-GC/MS enables the separation of fragments formed upon pyrolysis, thus revealing structural elements of the polymer. The flash pyrolysates of the purified megaspore walls contains p-vinyl phenol (8) as the most abundant component (Fig. 5.1A). p-Vinyl phenol is regarded to be a pyrolysis product derived from decarboxylation of p-coumaric acid moieties during pyrolysis (Wehling et al. 1993). Indeed, when pure p-coumaric acid is pyrolysed under the same conditions, p-vinyl phenol is generated as the major pyrolysis product (not shown). This is confirmed by DT/MS results of Boon et al. (1982) and Mulder et al. (1992). Thus, flash pyrolysis provides circumstantial evidence that the purified sporopollenin is composed of polymerised p-coumaric acid. However, due to the loss of important structural information, like the loss of carboxylic groups upon pyrolysis, detailed information on bonding sites and types within the polymer are difficult to deduce. The presence of benzaldehyde (3) and acetophenone (5) may point towards an ether linkage at the α position (α being the carbon next to the aromatic ring). 3-Methoxy-4-vinylphenol (9) is the decarboxylation product of 4-hydroxy-3-methoxycinnamic acid (ferulic acid) formed upon pyrolysis. This pyrolysis product indicates the presence of small amounts of ferulic acid monomeric units within the polymeric structure.
5.3.2. Py(TMAH)-GC/MS

To obtain more structural information an on-line methylating reagent was used during pyrolysis (Challinor 1989). This technique gives additional information to normal py-GC/MS. For example, carboxylic acids are detected as the corresponding methyl ester derivatives, whereas they may be decarboxylated under conventional conditions. Figure 5.2A shows the pyrolysate of the purified sporopollenin, however, this time treated with TMAH before pyrolysis. The resulting online methylation yields methylated p-coumaric acid (20) and, further decarboxylation to p-vinyl phenol does not occur (de Leeuw and Baas 1993). A small amount of methylated ferulic acid (21) and its derivative 1,2-dimethoxy-benzaldehyde (19) is also detected. TMAH treatment of pure p-coumaric acid supports this observation (Fig. 5.2C) and is consistent with the findings of Mulder (1992). The data confirm that p-vinyl phenol present in the ‘conventional’ flash pyrolysate (Fig. 5.1A) is generated from p-coumaric acid units present within the polymer.
Figure 5.3A: DT/MS of untreated crushed megaspores with mass spectrum of scans 56 towards 67 showing mainly triglycerides. B: DT/MS of purified sporopollenin showing mass spectrum of scans 49 towards 71 showing m/z 164, 120 and 107 which are all associated with the pyrolysis of the p-coumaric acid polymer.

5.3.3. DT/MS

Using pyrolysis GC/MS techniques, vital information may be lost if fragments are too large or too polar to pass through the capillary column of the gas chromatograph. Direct temperature-resolved mass spectrometry (DT/MS) enables the pyrolysis fragments to be introduced into the mass spectrometer resulting in the detection of
higher molecular weight pyrolysis products. Using this technique we might obtain an idea of the purity and uniformity or complexity of the sample. Because the sample is heated over a period of time, the total ion current (TIC) represents a temperature resolved disintegration of the polymeric matter and thermal extraction of extractable organic matter.

Figure 5.3A shows the DT/MS data of untreated crushed megaspores. The complexity of the sample is clear from the fact that the total ion current (TIC) shows 3 peaks plus a very broad signal. The MS summed spectra of the largest peak around scan 60 shows a mixture probably dominated by triglycerides of $C_{16}$ and $C_{18}$ fatty acids (of which the most important peaks are m/z 880, 854, 603, 576, 262 and 256). Figure 5.3B shows the DT/MS TIC of the purified sporopollenin. A single peak dominates around scan 60, indicating that the sample is homogenous in nature. The summed mass spectra show a strong m/z 164 and 120, which is the base peak of $p$-coumaric acid and $p$-vinyl phenol, its decarboxylated pyrolysis product respectively. Dimeric and trimeric structures of $p$-coumaric acid are not present in the pyrolysate. The triglycerides peaks are barely visible. This illustrates the necessity of a good sample cleanup, since $p$-coumaric acid is completely obscured in the pyrolysate of the pure megaspore, at the same time it illustrates that the triglycerides and fatty acids are not incorporated in the polymeric structure, since their abundance are significantly reduced upon cleanup.

These findings fit with DT/MS measurements from the bladders of *Pinus* pollen by Wehling et al. (1986), suggesting that sporopollenin from *Pinus* pollen bladders is essentially the same as *Isoëtes killipii* megaspore sporopollenin.

5.3.4. FT-IR

There is always doubt whether a pyrolysate actually reflects the bulk of the organic matter or not. FT-IR spectra of natural polymers are often difficult to interpret, but in this case FT-IR was used as an independent tool to check our pyrolytic data on the polymer. The spectrum of the purified sporopollenin shows a broad absorption at 3422 cm$^{-1}$ ($\nu$ O-H) indicating the presence of hydrogen bound OH groups. Medium absorptions at 2925 ($\nu_{\text{asymmetric C-H}}$) and 2860 cm$^{-1}$ ($\nu_{\text{symmetric C-H}}$) reveal characteristic bands of the methylene group originating from the $\alpha$ and $\beta$ position. A broad absorption at 1097 (including $\nu$ C-O and in plane $\delta$ C-H), at 781 ($\delta$ out of plane C-H) and 450 cm$^{-1}$ ($\delta$ aromatic C=C) indicate the aromatic bands, the in plane C-H and out of plane C-H bending and the C-C bending clearly stand out from the rest, pointing to a major aromatic structural unit. These FTIR data are additional evidence that the pyrolysis data indeed representative of the sporopollenin as a whole.

5.3.5. Ester-bound material

To further investigate the macromolecular structure of the sporopollenin, ester bound material released during base hydrolysis of the sample cleanup was analysed.
using GC/MS. The major compound released was p-coumaric acid (both cis and trans isomers). Hexadecanoic acid and octadecenoic acid, were the other major compounds released. Several minor components were detected, including 4-hydroxy-benzaldehyde, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, octadecanoic acid and octadecadienoic acid. All these components have been ester bonded to the sporopollenin. The purified sample (the resistant polymer) is completely devoid of these fatty acids. The released p-coumaric acid in this case is not C-O-C (ether) bound but could probably represent un-polymerised monomers of the sporopollenin which are ester bonded to available free carboxylic or hydroxy groups. Since only monomers and no dimers or trimers are found, it is likely that the polymeric structure of the sporopollenin does not include ester bonds and that the components identified after saponification represent only ester bound end members at the biopolymer.

5.3.6. Comparison with artificial dehydrogenation polymer of p-coumaric acid

All data of the purified sample are consistent with a biopolymer composed entirely of p-coumaric acid units. To further support this finding, we prepared an artificial poly-(p-coumaric acid) by polymerization of p-coumaric acid with peroxidase enzyme in an aqueous solution to form a dehydrogenation polymer, DHP, (van der Hage 1995; Hatcher and Minard 1996). Upon treatment with peroxidase enzyme and hydrogen peroxide, the p-hydroxy group of p-coumaric acid is dehydrogenated yielding a free radical. Although the yield was low, fluffy yellowish aggregations were obtained. This material was submitted to the same chemical treatments as the sporopollenin and the final residue was analyzed using the py-GC/MS and TMAH techniques.

It was not possible to saponify the DHP, which indicates that it doesn't contain esterified units of p-coumaric acids. The pyrolysates of the DHP (Fig. 5.1B and 2B) show the same components as that of the sporopollenin. Compounds 1, 2, 4, 6, 7 and 8 are present in both the pyrolystes of the actual sporopollenin sample and the DHP, suggesting that these are the pyrolysis products of β-O-4 linked p-coumaric acid units. The TMAH treated samples support this observation. The benzaldehydes that are lacking in the pyrolystes of the DHP, may be a result of the complete lack of α-O-4 links in the DHP, or at least suggest another bond type on the α position. Although it doesn’t seem to have α-O-4 bonds in contrast to the sporopollenin, it produces such similarities that the DHP must have a structure very closely resembling that of the sporopollenin.

5.4. Conclusions

The data shows that a pure sporopollenin from Isoëtes killipii C. Morton megaspores was obtained and, consisting of more or less exclusively of p-coumaric acid moieties cross linked through α-O-4 and/or β-O-4 linkages. Next to p-coumaric acid a trace amount of ferulic acid moieties are incorporated in the polymeric structure.
Van Bergen et al. (1993) have shown that p-coumaric acid was an important building block of the sporopollenin in Azolla and Salvinia micro and macrospores, while Wehling et al. (1993) showed that p-coumaric acid was a principal component of the bladders of Pinus pollen grains. We have shown here that sporopollenin from the macrospores of the heterosporous vascular cryptogam Isoëtes is essentially the same as the sporopollenin from the pollen grains of the gymnosperm Pinus. Sporopollenin from the angiosperm Corylus avellana was also shown to contain high amounts of p-coumaric acid (Herminghaus et al. 1988). This demonstrates that p-coumaric acid must be an important structural unit of sporopollenin and that the spores of cryptogams have the same sporopollenin component as the pollen grains from spermatophytes. However, the term sporopollenin is also erroneously used to refer to

Table 5.1 Identified pyrolysis products

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<th>ID nr.</th>
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<th>Molecular formula</th>
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<th>Base peak</th>
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biopolymers found in certain algae, fungi and resting stages of various microorganisms, the *p*-coumaric acid polymer described here clearly differs from these (Largeau and de Leeuw 1993; Blokker et al. 1999; Winkle-Swift 1998) and thus belongs to a different compound class. Sporopollenin of *Isoëtes killipii* used here is a non hydrolysable, non saponifiable component of the spore wall, which consists of *p*-coumaric acid units.

5.5. References


van der Hage (1995) Pyrolysis mass spectrometry of lignin polymers, PhD thesis University of Amsterdam, 156pp
