The Nature of Oxygen in Sporopollenin from the Pollen of *Typha angustifolia* L.

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Sporopollenin, *Typha angustifolia* L.

Native and peracetylated sporopollenin from the pollen of *Typha angustifolia* L. was investigated using several spectroscopic methods, inducing Fourier transform infrared spectroscopy (FTIR), solid-state ^{13}C-nuclear magnetic resonance spectroscopy (^{13}C-NMR) and X-ray photoelectron spectrometry (XPS). Interpretation of the experimental data shows that the greater part of oxygen found in sporopollenin originates from hydroxyl groups and must be derived from aliphatics and not from aromatics. This result indicates that not only aromatics and long unbranched aliphatics but also poly-hydroxyl aliphatic components are involved in the complex structure of the polymer. Furthermore, it is most probable that the monomers of the sporopollenin skeleton are linked by ether- and not by ester-linkage.

Two possible approaches are suggested for the characterisation of sporopollenin structure.

Introduction

Sporopollenin is a complex biopolymer present in the outer wall of spores and pollen. In the past, numerous investigations were carried out to elucidate the structure and chemical composition of sporopollenin. Different methods were used, such as immunocytochemical (Niester-Nyveld *et al.*, 1997), morphological (electron and X-ray microscopic analyses (Rowley *et al.*, 1995; Rowley, 1996 (further literature quoted herein); Thom *et al.*, 1998)) and chemical analyses of the structure (Schulze Osthoff and Wiermann, 1987; Guilford *et al.*, 1988; Herminghaus *et al.*, 1988; Espelie *et al.*, 1989; Hemsley *et al.*, 1993, 1996; Van Bergen *et al.*, 1993; Wilmesmeier *et al.*, 1993; Jungfermann *et al.*, 1997; Domínguez *et al.*, 1999; Blokker *et al.*, 1999).

Among the classical spectroscopic methods FTIR and ^{13}C CP/MAS NMR have played a dominant role in the determination of the chemical structure of sporopollenin. The interpretation of the spectral data reveals that the polymer contains varying amounts of aliphatic and aromatic constituents.

A further useful method for the analysis of sporopollenin is XP-spectroscopy which is a surface analytical tool that can supply information about the kind of elements present, their binding state and quantitative composition. Until now, this technique has been used only once for the investigation of sporopollenin obtained from different plants (Kawase and Takahashi, 1995).

Now that it is possible to dissolve sporopollenin in high concentrations (Jungfermann *et al.*, 1997), a variety of methods can be used for further analysis of this polymer. With the help of ^{1}H-NMR analysis of sporopollenin from *Typha angustifolia* L. and *Torreya californica* Torr. it was discovered that several aromatics are involved in this complex biopolymer (Ahlers *et al.*, 1999a;b).

This research centers on understanding the nature of oxygen in sporopollenin by investigating it in native and modified form with different spectroscopic methods.

Materials and Methods

Isolation, purification and modification of sporopollenin

For details about collection, isolation and purification see Ahlers *et al.* (1999a). Here, the extraction procedure was applied without acetone.

The peracetylated sporopollenin was obtained after the lyophilized native material was sus-

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pended in a 1:1 v/v $(CH_3CO)_2O$-pyridine solution and left at room temperature for 72h.

Fourier-transform-infrared spectroscopy (FTIR)

FTIR spectra were obtained using a Nicolet-5DXC-FTIR-Spectrometer equipped with the OMNIC 1.2-program (Nicolet, Offenbach, Germany) at the Institut für Organische Chemie der WWU-Münster. Samples were prepared by the KBr methods, spectra were extracted in the wave number region of 4000–400 cm^{-1}.

X-ray photoelectron spectrometry (XPS)

Investigations with XPS on electrically non-conductive samples involve the risk that the samples take on a positive charge due to the emission of photoelectrons generated by X-ray excitation. This charge can be almost completely neutralised by using a low energy electron flood gun. A more successful method is to avoid or reduce charging by a special sample preparation. This method involves suspending some micrograms of sporopollenin in 100 μl highly purified water. 20 μl of this suspension were pipetted onto a small piece of polished silicon wafer (as used in microelectronics) forming a drop of about 8 mm in diameter. This drop was dried under infrared light. The sample of sporopollenin prepared in this way formed a nearly closed monolayer of sporopollenin on the piece of the silicon wafer; this was verified by microscopy. These samples showed a residual conductivity and most of the emerging charge could be dissipated over the conductive silicon wafer.

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; *analyses*: take-off angle for the electrons 0° with respect to the surface normal, fixed analyser transmission (FAT) mode, pass energy of the concentric hemispherical analyser (CHA) 80 eV, data acquisition with 0.2 eV per step. A complete prevention of charge build-up on the samples was not possible.

^{13}C CP/MAS NMR spectroscopy

The ^{13}C-NMR anaylses were carried out on a Bruker DSX 500 spectrometer. Because of the small sample amount a special probehead with only 2.5 mm outer rotor diameter was used and the rotation rate was 14 kHz (for further details see Wilmesmeier *et al.*, 1993).

CP/MAS spectra as such are non-quantitative, however, the sample properties and the experimental conditions allow a semiquantitative interpretation of the obtained spectra.

Results

FTIR-data

The FTIR-spectra of the native and peracetylated sporopollenin from the pollen of *Typha angustifolia* are shown in Fig. 1. The absorption (Table I) in both spectra in the area of 3421 cm^{-1} represent hydroxyl groups and the signals at wave numbers 2932 cm^{-1}, 2858 cm^{-1}, 1436 cm^{-1} and 1373 cm^{-1} are due to aliphatic groups. The peak at 839 cm^{-1} is assigned to the out-of-plane C-H vagging vibration in aromatics. In the comparison of the two FTIR-spectra the following differences are conspicuous: the relatively high amount of hydroxyl groups in the native sporopollenin strongly decreases in the peracetylated sporopollenin. As expected, in the peracetylated sample the very intensive C=O stretching frequency appears at 1738 cm^{-1} (E), the stretching vibration (1243 cm^{-1} (I)) and the asymmetric bending vibration (1020 cm^{-1} (J)) derive from the C-O group and the two bending vibrations (1373 cm^{-1} (H) and 1436 cm^{-1} (G)) are due to the CH_3 groups. All the aforemen-

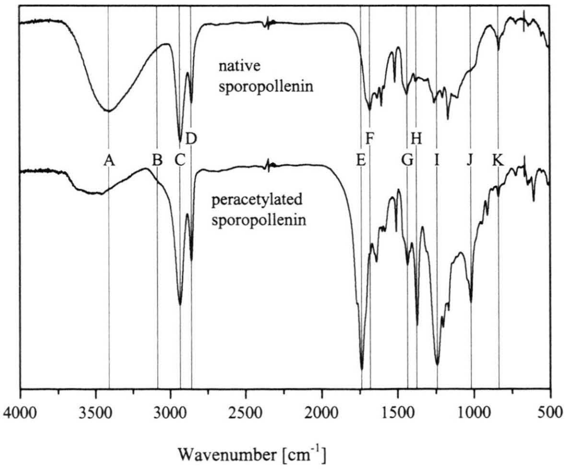

Fig. 1. FTIR-spectra of the native and modified sporopollenin.

Table I. Wave number and assignments for IR-bands of the sporopollenin samples (Hesse *et al.*, 1995).

Wavenumber [cm^{-1}]	Letter in Fig. 1	Assignment
3421	A	hydroxyl O-H
3100	B	aryl-protons often covered
2932	C	aliphatic C-H ν
2852	D	aliphatic C-H ν
1738	E	C=O ν from acetyl
1680	F	α,β unsaturated carbonyl group
1436	G	CH_3 δ
1373	H	CH_3 δs
1243	I	C-O ν from acetyl
1020	J	C-O δas from acetyl
839	K	C-H out of plane from aromatics

ν = stretching, δ = deformation, s = symmetric, as = asymmetric.

tioned signals are typical for acetyl groups. That not all of the hydroxyl groups were acetylated can be induced from the weaker intensity of the hydroxyl signal in the modified as compared with the native form.

Furthermore, it seems that the peak at 839 cm^{-1} (K) caused by the aromatic system is diminished in the spectra of the peracetylated sporopollenin in comparison with the corresponding peaks in the native preparation. The peak at 1680 cm^{-1} (F) in the spectrum of the native form does not suggest a normal carboxyl or ester linkage but rather an α,β unsaturated carbonyl group.

NMR-data

Figure 2 illustrates two solid state ^{13}C-NMR-spectra obtained from samples of native and peracetylated sporopollenin. Each distinct ^{13}C chemical environment gives rise to a peak at a characteristic chemical shift. The peaks can be loosely assigned according to spectral regions (Table II). In the spectrum of peracetylated sporopollenin two intensive peaks are present at 21 and 170 ppm in addition to the spectrum of the native sporopollenin. Both peaks are typical of acetyl groups. The peaks at 170 ppm are a marker for carboxylic groups and indicate the presence of carboxylic acids in native sporopollenin. Furthermore, the signal intensity in the range between 90 to 150 ppm is diminished in the peracetylated form in comparison to the native form.

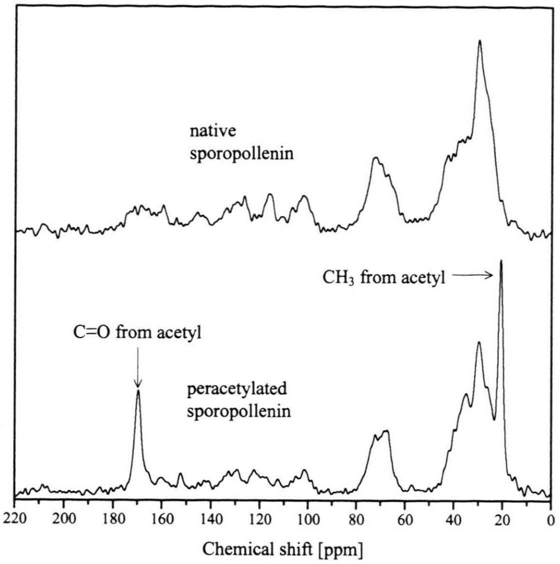

Fig. 2. Solid state ^{13}C-NMR-spectra of the native and modified sporopollenin.

Table II. Chemical shifts and assignments for ^{13}C-NMR signals of the sporopollenin samples (Hesse *et al.*, 1995, Love *et al.*, 1998).

Chemical shift [ppm]	Assignment
10–50	aliphatic hydrocarbons
50–90	oxygenated or nitrogenated aliphatic groups (ethers/alcohols)
90–160	unsaturated carbon environments (olefins/aromatics)
160–180	carboxylated groups (acids/esters)
170–205	aldehydes
175–225	ketones
170	C=O from acetyl
21	CH_3 from acetyl

Although in principle both aromatic and aliphatic hydroxyl groups can be peracetylated in this case it seems that aliphatic hydroxyl groups are the responsible ones for the peracetylation of the biopolymer, because only the integral of the peak at 170 ppm shown in Fig. 2 is similar to that of the hydroxyl groups which is only true for the integral of the peak around 70 ppm. The area of this pattern (70 ppm) is similar in both spectra (Fig. 2) and characteristic for oxygenated aliphatic components. However, the applied NMR-method can not distinguish between C-O shifts caused by hydroxyl, ether or ester linkages. Probably, this broad signal is the result of the merging of signals from various amounts of these functional groups.

The peaks in the range between 110 and 150 ppm can be assigned to bridgehead or protonated aromatic carbons and the signal around 160 ppm can derive from phenolic carbons in aromatic systems whereas the signal at 102 ppm can be caused by olefinic and/or aromatic constituents. The intensive signal at 29 ppm represents methylene groups which are constituents of long-chain unbranched aliphatics.

XPS-data

The wide-scan XP-spectra of native and peracetylated sporopollenin are given in Fig. 3. They show the photoelectron lines of carbon at 285 eV (C1s), of nitrogen at 399 eV (N1s) and of oxygen at 532 eV (O1s). The spectrum of the native sporopollenin shows an additional two silicon lines which originate from the silicon wafer. No further elements can be detected.

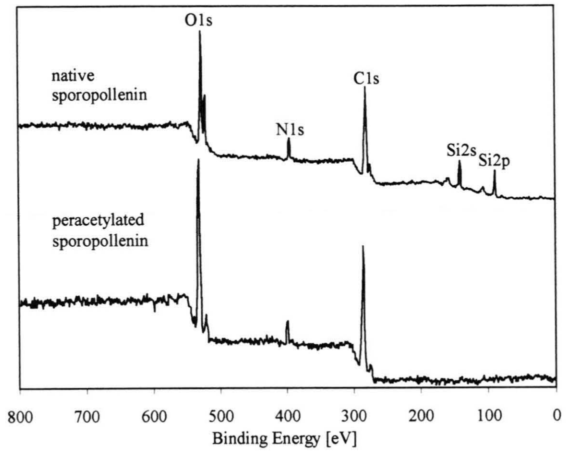

Fig. 3. Wide-scan XP-spectra of the native and modified sporopollenin.

For quantitative analysis, narrow-scan XP-spectra of the lines C1s, N1s and O1s were recorded using smaller energy steps and larger data acquisition times. These were evaluated by a standard procedure. The experimental data (dots) could be described by a curve (solid lines) which represents the summation of several Gaussian curves. The width of these curves depends on the width of the exciting X-ray line, the energy resolution of the analyser as well as the number of functional groups. If the binding energies of the functional groups are similar they will be described by the same curve. The maximum widths used were 2.6 eV for C1s, 3.5 eV for O1s and 2.3 eV for N1s, respectively. The energy positions of the described curves are a source of information about the chemical bonds.

Due to the fact that binding energy of different bonds as certained by the XPS-method can be very similar (see Table III), it is not always possible to say with certainty which functional groups are present in a complex substance such as sporopollenin. However, when information derived from the methods discussed above are pooled with the XPS-data, an accurate estimation of functional groups is possible.

The C1s spectra of both samples (Fig. 4) shows four bond-types of carbon; each type represents a definite functional group (Table III). The major peak (1) at 285.0 eV represents C-C and/or C-H bonds, the peak (2) at 286.45 eV represents C-O-C and/or C-OH bonds, the peak (3) at 287.9 eV can be caused by O-C-O and/or C=O bonds whereas the peak (4) at 289.0 eV is assigned to O=C-O structure elements.

The O1s peaks of the XP-spectra (Fig. 5) from the samples can be described by three Gaussian curves. The smallest peak (5) is probably due to charging effects and will therefore not be considered. For the interpretation of peak 6 and 7 the results of the FTIR- and the NMR-methods

Table III. Normalized concentrations for carbon in different functional groups of the two samples extracted from XPS C1s spectra by fitting procedure.

Type	Bond	BE[1]	BE[2]	Sporopollenin nativ [at-%]	Sporopollenin peracetylated [at-%]
(1)	C-C C-H C-N	285.00 285.00 285.94	285.00	61 ±2	52 ±2
(2)	C-O-C C-OH O=C-O-C*	286.45 286.55 286.64	286.45	22 ±2	27 ±2
(3)	O-C-O C=O O-C-N O=C-N	287.9 287.9 287.8 288.1	287.9	14 ±2	7 ±2
(4)	O=C*-O-C O=C-OH	289.0 289.3	289.0	3 ±2	14 ±2

[1] Mean binding energy (BE) [eV] of functional groups relative to saturated hydrocarbon (C1s = 285.0 eV), (Beamson and Briggs, 1992).

[2] BE values used in this work for curve fitting.

* denotes the C atom for which the BE is given.

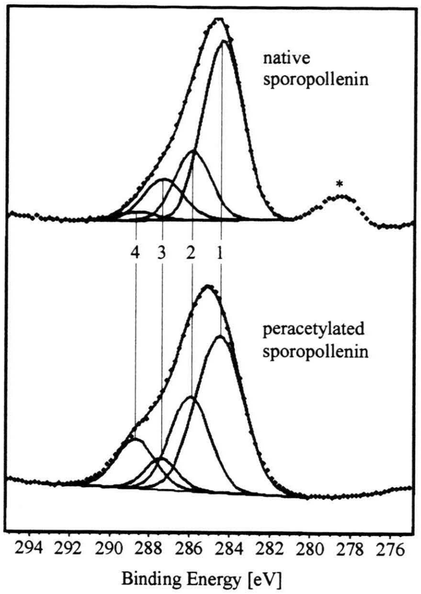

Fig. 4. XP-spectra of the C1s energy region of native and modified sporopollenin (dots) and the fit curves (solid lines). The peak indicated by an asterisk can be attributed to hydrocarbon contaminations of the surface of the silicon wafer.

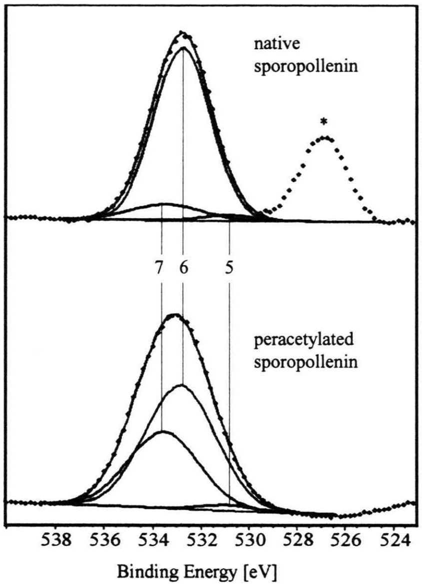

Fig. 5. XP-spectra of the O1s energy region of native and peracetylated sporopollenin (dots) and the fit curves (solid lines). The peak indicated by an asterisk can be attributed to the slightly oxidized surface of the silicon wafer.

should be considered. The NMR-spectroscopy of the samples (Fig. 2) shows that great amounts of oxygen must be single bonded to aliphatic carbons and that the content of C=O groups from acids or esters is low. Furthermore, the interpretation of both FTIR- and NMR-spectra (Fig. 1 and 2) show that peracetylation was successful. The peak (7) at 533.6 eV (Fig. 5) must be assigned to the carbonyl oxygen (C=O) from the carboxyl group due to the dramatic increase of this peak in the spectra of the peracetylated sporopollenin (Fig. 5).

The most intensive peak (6) at 532.9 eV is due to single bond aliphatic oxygen-carbon bonds (C-O) from ethers and/or alcohols; this assignment accords with the decrease of this peak after peracetylation (Fig. 5). Analyses of the data from Fig. 5 shows that the native form contains 88 at-% C-O and 10 at-% C=O while the peracetylated form contains 61 at-% C-O and 37 at-% C=O, respectively. Both the increase of the C=O signal and the fact that not all of the hydroxyl groups are peracetylated (see Fig. 2 signal A in the peracetylated form) indicates that the main part of peak 6 is due to the hydroxyl groups of alcohols. However, it is possible that apart from alcohols a minor part of peak 6 is caused by ether or ether-like structure elements.

Figure 6 illustrates the N1s line of the XP-spectra of both samples. The peak (9) at 399.9 eV originates from primary or secondary amines. The second peak (8) at 398.3 eV, which is only found in the peracetylated sporopollenin still not be assigned to a definite bond.

Nitrogen was found with the help of two different methods.

In the Table IV the summarized results of the XPS and elemental analyses are shown. The values

Table IV. Chemical composition of native and peracetylated sporopollenin according to elemental analyses and calculated without hydrogen. Data in parentheses according to XPS measurements.

Element	Sporopollenin native [at-%]	Sporopollenin peracetylated [at-%]
C	74.6	73.6
C	(65)	(66)
O	24.1	26.1
O	(27)	(29)
N	1.3	0.3
N	(8)	(6)

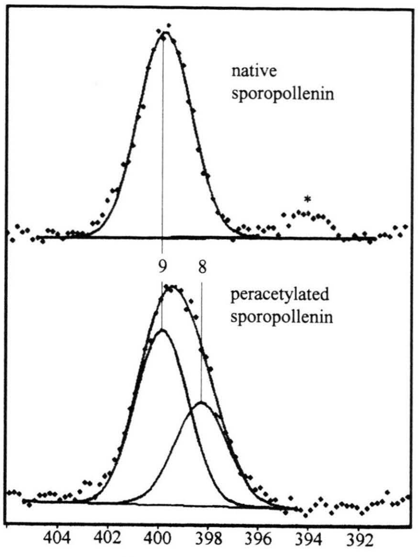

Fig. 6. XP-spectra of the N1s energy region of native and peracetylated sporopollenin (dots) and the fit curves (solid lines). The peak indicated by an asterisk can be attributed to nitrogen-containing contaminations of the surface of the silicon wafer.

are given in at-% and conform with those described by Jungfermann *et al.* 1997. Our assumption is that the low N content is due to the contamination of nitrogen during the purification procedure in which two enzymes (see part material and methods) are used.

Because XPS-data gives only the chemical composition of the sample surface and not that of the whole sample a direct comparison of the results obtained from elemental analyses and XPS is not possible. The data of both methods do show the same trends of chemical elemental composition: oxygen increase, a constant carbon and a nitrogen decrease.

Furthermore, the peracetylated sporopollenin differs from the native preparation in more than the peracetylation; other surface reactions are possible. For example, peripheric parts of the biopolymer can dissolve during the reaction or transformation processes can be caused by the modification.

For this reason the applied gravimetric analysis, which shows a 20% increase of the peracetylated sporopollenin in comparison with the native polymer, cannot be used to calculate the absolute content of peracetylated hydroxyl groups.

However, the interpretation of the XP-spectra in combination with that of the FTIR and solid state ^{13}C-NMR uncovers two crucial points. First, peracetylation has taken place and second, two or three oxygen bond types are involved in the native sporopollenin. The major oxygen is found in aliphatic hydroxyl groups (Fig. 5, peak 6), some is found in ester groups (Fig. 5, peak 7) and a little could possibly be a part of aliphatic ether or ether-like bonds (low contribution of peak 6 in Fig. 5).

Discussion

The combined use of several different methods to investigate the structure of the native in comparison to the peracetylated polymer allowed us to gather detailed information about the nature of oxygen in sporopollenin. It is surprising that, in the last decades, very little attention has been paid to the hydroxyl groups, although in the FTIR-spectra their presence is obvious (Wilmesmeier *et al.*, 1997; Domínguez *et al.*, 1999).

Particularly during the oxidative degradation for example with ozone (Domínguez *et al.*, 1999) or ruthenium tetroxide (Blokker *et al.*, 1999) the oxidation of hydroxyl groups is very probable and other side reactions can not excluded in this complex biopolymer (Bailey, 1958; Razumovskii and Zaikov, 1984). Only Blokker *et al.* (1999) do suggest that it is not possible to distinguish between original acid groups and primary hydroxyl groups or between single ether-linkage and secondary hydroxyl groups. In their model of algaenan (sporopollenin-like material) obtained from the green alga *Chlamydomonas monoica*, the authors proposed that C22, C24 and C26 monomers are connected by ether- or esters-links.

On the basis of our results we assume that ether-linkage are dominant in the sporopollenin skeleton, because acid groups are present only in minor amounts. The most convincing argument which favours ether-linked bonds is that despite several attempts no one has been able to cleave sporopollenin to its monomers.

Furthermore, it is evident that the acetolysis by Erdtman (1960) leads to a similar modification as does the peracetylation described in this paper (Hemsley *et al.*, 1996). The advantage of the peracetylation is its mildness. Additionally the native sporopollenin is closer to the genuine state than the biopolymer yielded after the method described by Erdtman.

It is difficult to compare results of different structural analyses of other groups because they use material which is obtained from plants or algae, and in addition they applied different isolation and purification procedures (Erdtman, 1960; Shaw and Yeadon, 1966; Baldi *et al.*, 1987; Southworth *et al.*, 1988; Schulze Osthoff and Wiermann, 1987; Jungfermann *et al.*, 1997; Domínguez *et al.*, 1998).

In order to reveal the structure of sporopollenin two approaches can be used. The best approach is the solubilisation of the biopolymer to make it amenable to ^{1}H- and ^{13}C-NMR techniques, ^{1}H-NMR investigations of *Typha angustifolia* L. and *Torreya californica* Torr. (Ahlers *et al.*, 1999a;b) have already been carried out. Enzyme-based purification method has the advantage that the sporopollenin obtained is soluble in 2-aminoethanol.

Secondly, the gentle cleavage of the ether-linkage is achieved through the use of a modification to protect the active hydroxyl groups proceeded by a selective cleavage. The recently described method "DFRC" (derivation followed by *reductive* cleavage from Lu and Ralph (1997a) can also be a successful approach). This is an efficient method for the selective cleavage of α- and β-aryl ethers from lignin which releases monomers for characterisation (Lu and Ralph, 1997b; Lu and Ralph, 1998). However, it must be done very carefully to avoid side reactions which can alter the structure of the native biopolymer.

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