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High Resolution Solid State ^{13}C NMR Spectroscopy of Sporopollenins from Different Plant Taxa

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ABSTRACT

The extremely chemically resistant component of the cell wall of spores, pollens, and some microorganisms, sporopollenin, is generally accepted to be derived from carotenoids or carotenoid esters. However, we report here that ^{13}C NMR analyses of sporopollenin from several sources shows that this widely held view is incorrect, with one possible exception. Sporopollenin is not a unique substance but rather a series of related biopolymers derived from largely saturated precursors such as fatty acids. The biopolymers contain widely varying amounts of oxygen in the form of ether, hydroxyl, carboxylic acid, ester, and ketone groups.

The outer cell wall, or exine, of many spores, pollens, and certain microorganisms is made of sporopollenin. The extremely high chemical and physical stability of sporopollenin accounts for the survival of ancient pollens over millions of years with full retention of morphology (3). However, this same resistance to environmental influences has made the elucidation of the structure of sporopollenin a formidable problem. Not only is the polymer chemically unreactive, it is also insoluble in both aqueous and organic solvents. On the basis of elemental analysis, Zetzsche et al. (14) reported that sporopollenin was an oxygenated hydrocarbon having a chemical composition between $C_{90}H_{134}O_{20}$ and $C_{90}H_{150}O_{33}$. Brooks and Shaw (2) later suggested that sporopollenin could be formed by the oxidative polymerization of a mixture of carotenoids and carotenoid esters. These authors noted that the concentration of carotenoids increased 100-fold during zygospore formation in the fungus *Mucor mucedo*. In addition, ^{3}H labeled β-carotene incorporated into the sporopollenin of several fungi and ^{14}C labeled carotenoids incorporated into the pollen of young *Lilium* and *Curcurbita* plants. They also observed that there was a chemical similarity between polymerized carotene and sporopollenin isolated from *Lycopodium*. Other studies, however, provided conflicting evidence and suggested alternative monomers. Inhibitors of carotenoid biosynthesis had little effect on the formation of the sporopollenin coat in pollen from *Curcubita pepo* (10). Spectroscopic analysis of the chemically stable material in the outer cell wall of the alga *Botryococcus braunii* indicated that the material contained some decidedly noncarotenoid features, such as long alkyl chains, few methyl groups, a low level of unsaturation, and cis double bonds (1, 6). Further, phenylalanine was found to incorporate into the sporopollenin fraction in developing tulip microspores (11). The chemical composition of sporopollenin is thus far from clear. To remove the confusion as to the nature of this important plant material, we have analyzed sporopollenin samples gathered from a broad phylogenic range of plant sources using high resolution solid state NMR spectroscopy. This technique is capable of identifying the chemical state of carbon nuclei in intractable substances such as cellulose, lignin, and other plant and fossil materials (5).

MATERIALS AND METHODS

Sample Preparation. Samples of wheat (*Triticum aestivum* L.) pollen were collected with the aid of a hand held vacuum cleaner from fields located in Davis, CA in March 1986. Pine (*Pinus thunbergiana* L.) pollen was harvested with the aid of a vacuum cleaner from trees growing in Danville, CA in February 1986. Corn (*Zea mays* L.) pollen was isolated from greenhouse plants grown at Sogetal in Hayward, CA. Ragweed (*Ambrosia trifida* L.) pollen was purchased from Sigma. Purified *Lycopodium* (L.) sporopollenin, which was isolated using the procedure originally described by Zetzsche (13), was purchased from Polysciences (Warrington, PA). Cells of *Chlorella fusca* (L.) were provided by Dr. Scott Pore (West Virginia University Medical School).

All the samples were treated according to the nonoxidative, extraction-hydrolysis method of Zetzsche (13) which yielded sporopollenins having the same morphology as the starting pollen grain. The collected pollen was suspended in ethanol and separated from anthers and other contaminates using a Buchner funnel as a sieve. The purified pollen in a Soxhlet thimble was placed in a Soxhlet extractor and washed with boiling ethanol until a clear wash was obtained. This extraction procedure was repeated with hot ether, and the resulting solid was dried at 50°C in a vacuum oven. A suspension of the washed pollen grains in a 6% aqueous potassium hydroxide solution was heated at reflux for 6 h with vigorous mechanical stirring. The suspension was neutralized and the hollow pollen grains were isolated by either filtration or centrifugation, washed with hot water (3 times), hot ethanol (3 times), and hot ether (3 times), and dried at 50°C in a vacuum oven. A suspension of this powder in 85% phosphoric acid was stirred vigorously at 30°C for 6 d. The mixture was diluted with an equal volume of water and centrifuged (15 min, 3000 rpm). The top layer containing sporopollenin was removed. The dark brown solid was washed three times with hot water, three times with hot ethanol, and three times with ether. The resulting light tan material was dried in a 50°C vacuum oven prior to the NMR experiments.

NMR Spectroscopy. Complete ^{13}C NMR spectra of the sporopollenins were obtained at 37.8 MHz on a home-built double resonance spectrometer. The ^{13}C magnetization was developed via cross-polarization (9) with a mix time of 2 ms. Proton decoupling was applied during the 12.8 ms data acquisition period. The powder samples were spun at the magic-angle (12) at 2.7 kHz in a saphire rotor with Kel-F end caps. The ^{13}C NMR spectra of sporopollenin with resonances from only the nonproton bearing carbons and methyl carbons (Selpen spectra) were obtained as described above with the addition of a 75 μs pulse

delay after the end of the mix pulse and prior to the application of proton decoupling and data acquisition (8). Each spectrum is the result of 10^4 transients and is expanded vertically threefold. Chemical shifts are expressed in parts per million (ppm) and are relative to external tetramethylsilane.

RESULTS AND DISCUSSION

The carbon resonances in sporopollenin spectra occur primarily in four distinct regions but vary in peak intensity and shape (Fig. 1, A–E). For example, the complete spectrum of sporopollenin obtained from the alga *Chlorella* (Fig. 1A) has a strong aliphatic carbon absorbance between 20 and 40 ppm, a weaker resonance at about 70 ppm indicative of a carbon bearing oxygen, an olefinic resonance centered at 130 ppm, and a carbonyl resonance at 170 ppm indicative of an ester or a carboxylic acid. In addition to these four kinds of NMR signals, a fifth type of resonance at 206 ppm was observed in the spectrum of sporopollenin from *Lycopodium* spores (Fig. 1B). This chemical shift is suggestive of a ketone carbonyl carbon. The *Lycopodium* spectrum also shows that the moss material contains significantly more unsaturation than any other sample studied. The pine spectrum (Fig. 1C) has less carbonyl intensity than other sporopollenins. In addition, pine pollen sporopollenin differs from the material isolated from alga or *Lycopodium* in the amount of oxygen present as noted by the increase in the resonance centered at 70 ppm and the shoulder on the aliphatic carbon resonance at about 40 ppm. The resonance at 40 ppm is attributed to carbon adjacent to an oxygen-bearing carbon. The spectra of sporopollenin from wheat and ragweed pollen (Fig. 1, D and E) are quite similar to each other. These spectra contain each of the five resonance types noted above, but in different relative intensities than the other sporopollenins investigated.

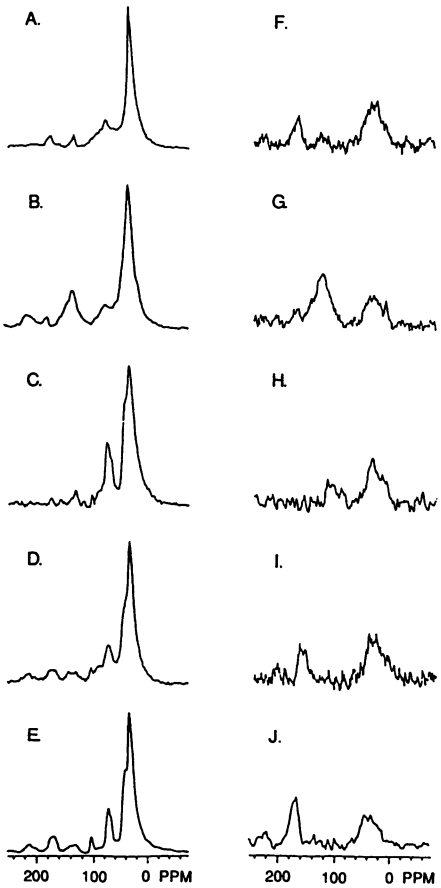

FIG. 1. ^{13}C magic angle spinning NMR spectra of sporopollenins obtained at 37.8 MHz. A and F, *Chlorella*; B and G, *Lycopodium*; C and H, pine; D and I, wheat; E and J, ragweed. A–E are full spectra. F–J are Selpen spectra containing resonances only from carbons weakly coupled to protons. The Selpen spectra are expanded 3-fold vertically.

Further information on the types of carbons found in the different sporopollenins was obtained using the Selpen pulse sequence (8) which suppresses signal from carbons bearing hydrogens (except methyls), *i.e.* only nonprotonated carbons and methyls are observed (Fig. 1, F–J). The Selpen spectrum of *Chlorella* (Fig. 1F) indicates that few if any olefinic carbons are fully substituted since the signal at 130 ppm is suppressed. However, the intensity at 170 ppm survives as well as some of the signal in the aliphatic region. This is consistent with the presence of carbonyl carbons and strongly suggestive of quarternary aliphatic carbons and perhaps methyl groups. The *Lycopodium* spectrum (Fig. 1G), by contrast, retains significant olefinic intensity as well as a some of the aliphatic resonance and a clear methyl signal. The retention of a large portion of the olefinic signal is consistent with the presence quaternary olefinic carbons and a monomer of terpene or carotenoid origin, which separates *Lycopodium* from the other samples examined. The spectra of the sporopollenin from pine (Fig. 1 H) also retains aliphatic signal and has a small absorbance at about 120 ppm. As in the case of the full spectra, sporopollenin from wheat and ragweed pollen give nearly identical Selpen spectra (Fig. 1, I and J). These spectra contain only resonances for aliphatic and carbonyl carbons.

A comparison of the spectra shows two trends as one moves from alga to moss to the higher plants: broader resonances and an increase in the intensity of resonances due to oxygenated carbons. The increase in the peak width indicates that a less homogeneous and more complex biopolymer forms the exine layer. This conclusion is supported by the increase in the amount and the variety of oxygenated carbons in the biopolymer. It is also possible that oxygenated aromatic rings are present at low levels in sporopollenins. Spectra of lignin, a material which contains such moieties, contains resonances between 110 and 160 ppm although in a different intensity ratio than that observed in sporopollenin (7). This point is of further interest because of the report that radiolabeled phenylalanine is incorporated into sporopollenin (11). In addition, phenolic compounds can act as cross-linking agents in other plant cell wall components (4).

The high resolution ^{13}C NMR spectra obtained from the chemically resistant exine of different plant classes indicate that these materials are all distinct substances. Sporopollenin is therefore a class of biopolymers rather than a single, homogeneous macromolecule. The appearance of the spectra is more supportive of a fatty acid precursor (long saturated aliphatic chains, low olefinic intensity) than of a carotenoid precursor (significant quaternary olefinic intensity and methyl intensity). Indeed, only the spectrum from the *Lycopodium* spores showed a substantial similarity to that from polymerized β-carotene (spectrum not shown).

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