Organic chemical differentiation within fossil plant cell walls detected with X-ray spectromicroscopy

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ABSTRACT

Organic matter preserved in cell walls of permineralized plant fossils was analyzed by using scanning transmission X-ray microscopy and spectroscopy at energies near the 1*s* absorption edge of carbon. Microchemical analyses were performed directly on cellulose acetate peels of the fossils, preserving information on the anatomical distribution of organic materials. Individual tracheid walls in both Eocene and Early Devonian fossils exhibit spatially distinct chemical zoning inherited from original wall biopolymers and cell-wall microstructure. Molecular analysis of submicrometer domains using carbon X-ray absorption near-edge spectroscopy documents the differential distribution of hydroxylated aromatic and alcohol (and/or ether) carbon in the inner and outer regions of tracheid walls. This zonation reflects the deposition of lignin and structural polysaccharides in Devonian plants, indicating biochemical and developmental pathways similar to those of living tracheophytes.

Keywords: organic geochemistry, X-ray spectroscopy, lignin, cellulose, paleobotany, taphonomy.

INTRODUCTION

Permineralization preserves plant fossils in three-dimensional cellular detail. This circumstance has long afforded opportunities for the study of anatomical evolution, but it also raises the possibility that cell- and tissue-specific chemical analyses might provide insights into plant biochemical and physiological evolution. However, the amount of carbon preserved in permineralized fossils is generally quite low (Boyce et al., 2001), requiring that relatively large samples be demineralized to obtain sufficient material for organic geochemical analysis (Collinson et al., 1998; Edwards et al., 1997; Hemsley et al., 1996; Niklas and Pratt, 1980). Anatomical context is lost in the process, which is problematic because fossil organic matter has typically been altered substantially during diagenesis. In the absence of anatomical reference points to guide comparisons with living plants, correlations between preserved molecular structures and original plant biomolecules may be tenuous.

Recent advances in X-ray microfocusing techniques coupled with brilliant, synchrotron-derived X-ray sources have led to the development of soft X-ray spectromicroscopy that allows for the analysis of functional-group distributions in bioorganic structures at spatial resolutions approaching 50 nm (Cody, 2000; Jacobsen and Kirz, 1998). In this paper we describe the application of carbon (1*s*) X-ray absorption near-edge spectroscopy (C-XANES) to characterize the organic chemistry within structurally differentiated regions of tracheid cell walls in both recent and fossil plants. These include the extant red cedar *Juniperus virginiana*; *Metasequoia milleri*, a conifer from the middle Eocene (ca. 40 Ma) Princeton Chert (Basinger, 1981); and the stem lycopod *Asteroxylon mackiei* from the Lower Devonian (ca. 400 Ma) Rhynie Chert (Kidston and Lang, 1920). The high sensitivity of X-ray spectromicroscopy permits analysis of the small amounts of organic matter extracted by cellulose acetate peels of the fossils (Joy et al., 1956), thereby allowing demineralization while maintaining the anatomical context of the preserved organic chemistry (Fig. 1). The ability to apply these techniques to peels means that a vast reservoir of information on the micrometer-scale distribution of organic chemistry within fossil plants is already available in museum paleontological collections.

SCANNING TRANSMISSION X-RAY MICROSCOPY

The power of soft X-ray microscopy is that image contrast is based on chemistry through the use of a high-resolution monochromator (~0.03 eV) (Jacobsen and Kirz, 1998) tuned to the characteristic energy of specific core level (1*s*) to bound-state (e.g., π^*) electronic transitions. For the modern wood (Fig. 2A), an image was acquired at 285 eV, where X-ray absorption results from the excitation of a core electron up to a low-energy, unoccupied, π^*, molecular orbital of aromatic (and possibly olefinic) carbon. Tracheids display a high degree of chemical differentiation within their cell walls that reflects variation in the distribution of specific cell-wall biopolymers such as lignin and cellulose (Sjöström, 1993). Because lignin is the only cell-wall component that is aromatic, the differential absorption of 285 eV X-rays, displayed as the gray scale in

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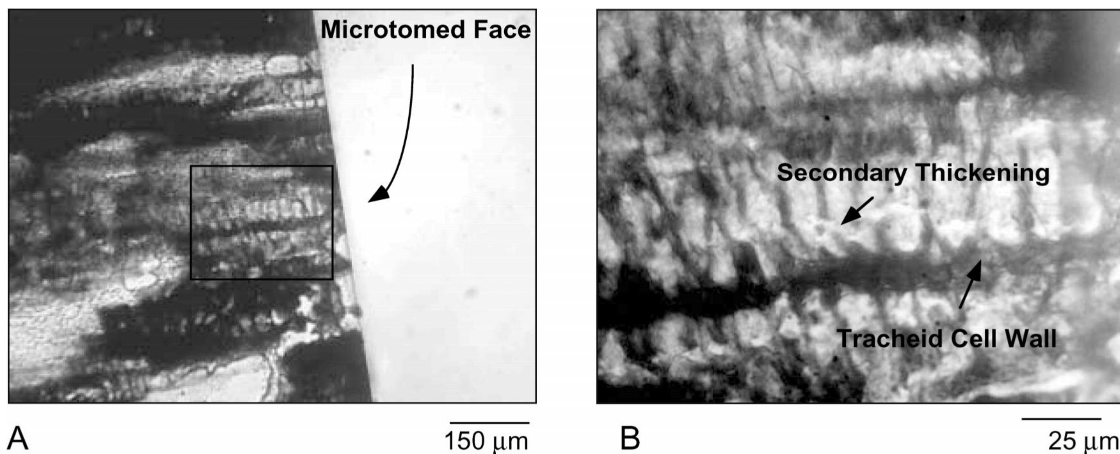

Figure 1. Optical microscopic images of cellulose acetate peel of Early Devonian *Asteroxylon* fossil embedded in epoxy. A: Low-magnification image showing cut edge from which samples for X-ray spectroscopic analysis were taken. B: Higher-magnification image revealing anatomical detail of tracheid cell walls with secondary thickenings.

Figure 2A, indicates the distribution of lignin within individual tracheid walls.

For the fossil specimens, analyses were performed on cellulose acetate peels of fossils that were embedded in epoxy and sectioned, at right angles to the peel surface, to the thickness of 70–150 nm required for X-ray transmission (Fig. 1). This section-of-a-section approach naturally limits image acquisition to small parts of the fossil organic matter. The integrity of the fossil samples is somewhat degraded by compression of the fragile demineralized organic matter during peel preparation. However, despite mechanical damage, precise control is maintained over which cell-wall material is analyzed (as seen in the optical image in Fig. 1). X-ray images were acquired at 286 eV (Fig. 2B) and 286.3 eV (Fig. 2C) to highlight the fossil organic matter surrounded by both epoxy and cellulose acetate. The Eocene tracheids (Fig. 2B) exhibit intact cell-wall structure, with zoned variation in absorption intensity across the cell wall. Similar to the Eocene sample, collapsed *Asteroxylon* tracheids (Fig. 2C) reveal a distinct outer domain characterized by strong X-ray absorption and an inner domain with weaker absorption.

X-RAY ABSORPTION NEAR-EDGE SPECTROSCOPY

C-XANES spectra were acquired for quantitative organic analysis of the discrete cell-wall regions in each sample (Fig. 3). The C-XANES spectra of different regions of the *Juniperus* tracheid wall reveal absorption bands corresponding to the $1s$-π^* transition of aryl-C,H (at ~285 eV) and the $1s$-π transition of aryl-O (at ~287 eV) that are characteristic of lignin. At ~289.5 eV, a third strong broad band corresponds to a C-H $1s$-σ^* transition associated with oxygen-substituted sp^3-hybridized carbon, C_{Al}-O, of the alcohol and ether carbons found in lignin as well as polysaccharides such as cellulose and hemicellulose. The differences in the two spectra reflect variation in the proportions of lignin and polysaccharides. The proportion of lignin and structural polysaccharide was determined by fitting the C-XANES spectra using pure lignin and cellulose spectra. For example, the middle lamella of *Juniperus* tracheids is composed of 75% lignin and 25% polysaccharide, and the inner region of the secondary wall is composed of 48% lignin and 52% polysaccharide. Typical C-XANES analyses of inner regions of mature tracheid cells often reveal polysaccharide concentrations of >70% (Cody, 2000).

For both fossils, C-XANES reveals preservation of regional differences in carbon chemistry (Fig. 3). The C-XANES spectra of inner and outer regions of the Eocene tracheids both exhibit pronounced absorption bands corresponding to aryl-C,H and aryl-O, while exhibiting diminished intensity in the C_{Al}-O absorption band (compared with modern *Juniperus*). The cell-wall regions differ in their chemistry, however, in that the outer region has a higher overall concentration of aromatic carbon, whereas the inner region has a sharp absorption band at ~288.5 eV, corresponding to a $1s$-π^* transition of carboxyl groups. The C-XANES spectra of *Asteroxylon* tracheids exhibit a broader $1s$-π^* transition-peak width, indicating greater heterogeneity among aromatic groups, e.g., a wider range of aryl substitutions. Similar to the younger samples, however, the outer region of the *Asteroxylon* tracheid wall has proportionally more aromatic carbon than the inner region. The inner region exhibits proportionally greater absorption at 288.5 and 289.5 eV, indicating a greater abundance of COOH and C_{Al}-O relative to the outer region. No infiltration by cellulose acetate or epoxy that might alter the C-XANES spectra of the fossil organic matter has been observed. If even a small amount of epoxy or cellulose acetate contributed to the absorption spectra of the fossil organic matter, this would be readily detected (and could be easily removed by spectral subtraction) because the characteristic absorption bands of the simple cellulose acetate and bisphenol epoxy molecules are intense and narrow relative to the chemically variable fossil organic matter.

In order to compare quantitatively the chemistry within the different regions, a self-consistent fitting scheme was used to extract relative absorption-band intensities. The C-XANES spectra were fit with Gaussian bands at 285.2 eV (aryl-C,H), 287.1 eV (aryl-O), 287.9 eV (C_{Al}-H), 288.5 eV (carboxylate), 289.5 eV (C_{Al}-O), and 291 eV (absorption associated with the ionization edge). Because the molar absorption cross section differs among organic functional groups (Hitchcock et al., 1992; Francis and Hitchcock, 1992; Ishii and Hitchcock, 1988), each absorption band must be normalized in intensity to obtain representative functional-group concentrations. Compositional estimates were made by using scaled intensities based on published spectra of known compounds (Cody, 2000; Ade, 1998; Hitchcock et al., 1992; Ishii and Hitchcock, 1988); these estimates are presented as the number of carbons of a given functional group normalized to six aromatic carbons, i.e., a single aromatic ring (Table 1). The principal trends with increasing sample age are a progressive increase in the fraction of aromatic, carboxyl, and aliphatic carbon and a reduction in the fraction of aryl-O and C_{Al}-O. Preserved chemistry differs from sample to sample, reflecting variations in diagenetic history. Nonetheless, the spectral signatures

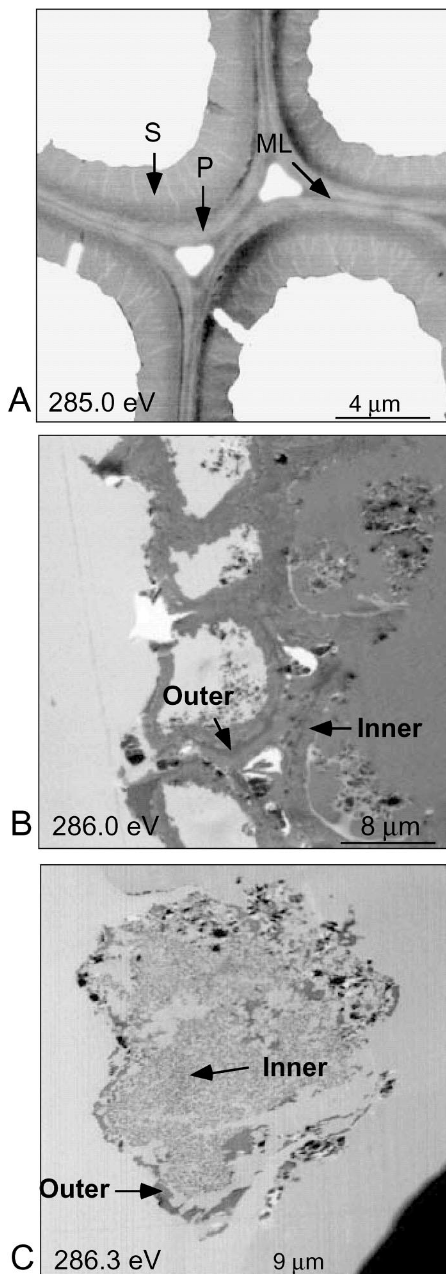

Figure 2. High-resolution, soft X-ray, gray-scale images of tracheids from ultrathin sections of (A) extant and (B, C) fossil plants. Increased absorption intensity (shown as darker areas) is due to presence of aromatic carbon. In images of fossil material (B, C), tracheids are at interface of cellulose acetate of peel on left and epoxy in which peel was embedded on right. Epoxy and cellulose have sharp, intense absorption bands at 285 eV and 288.5 eV, respectively. A: Extant *Juniperus* wood. Lignified middle lamellae connective between adjacent tracheids (ML), unlignified primary wall (P), and both lignin-rich and lignin-poor zones of secondary cell wall (S) are well resolved. B: Peel of Eocene *Metasequoia* wood. Within tracheid cell walls, absorption contrast reveals distinct outer region (strong absorption), inner region (weaker absorption), and very dark silica particles. C: Peel of Devonian *Asteroxylon* stem vasculature with sample taken along microtomed face depicted in Figure 1A. Tracheids reveal mechanically resistant, strongly absorbing outer region and weakly absorbing inner region that was more easily crushed during sample preparation.

shown in Figure 3 are consistent with a common underlying biology, i.e., concentric zonation in secondary-wall chemistry imparted largely by developmental variation in the ratio of lignin to cellulose and other polysaccharides deposited during cell-wall formation.

DISCUSSION

The observation of a chemically differentiated cell wall, even in the 400 Ma sample, demonstrates that carbon derived from both lignin and structural polysaccharides has been preserved within individual tracheids, albeit in a diagenetically transformed state. If only lignin-derived carbon remained, any molecular signature of chemical zoning in the cell wall would be lost.

Earlier studies have interpreted the organic matter preserved in fossils as either derived from complex polymerization reactions between original biomolecules (Briggs, 1999) or derived from the selective preservation of a limited range of biochemical materials (Tegelaar et al., 1989). For example, the aliphatic component of organically preserved fossils has been interpreted as derived from the diagenetic polymerization of plant and animal cuticles (Stankiewicz et al., 2000; Briggs, 1999; Collinson et al., 1998), whereas the diverse suites of aromatic compounds found in fossiliferous rocks typically have been interpreted as derivatives of lignin and perhaps other phenolic biomolecules (Edwards et al., 1997; Niklas and Pratt, 1980). Our study demonstrates that the aromatic carbon present in fossilized plants may be derived from originally nonaromatic biopolymers, such as polysaccharides, and that these diagenetic reactions occur in place at a submicron scale. Because of the extremely fine spatial scale of this recruitment of biological compounds into the polymerization reactions, the diagenetic products can retain a signature of the original lignin and cellulose distribution despite extensive chemical alteration. The present results required the coupling of traditional paleontological observation of anatomy with a novel spectroscopic instrument capable of submicrometer-scale analysis. In combination, the anatomical detail afforded by acetate peels and the microanalytical precision of scanning transmission X-ray microscopy offer substantial new possibilities for understanding the biology of long-extinct fossils.

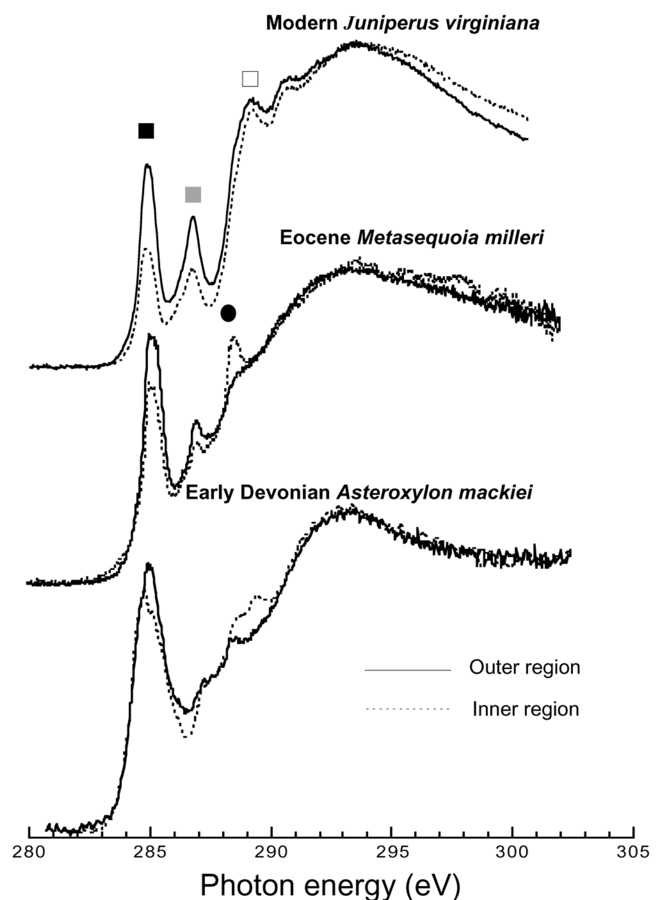

Figure 3. Carbon X-ray absorption near-edge spectroscopy spectra of inner (dashed line) and outer (solid line) regions of tracheids from modern *Juniperus*, middle Eocene *Metasequoia*, and Early Devonian *Asteroxylon*. Absorption bands result from 1*s*-π* transition of aromatic carbon (black square), 1*s*-π* transition of oxygen-substituted aromatic carbon (gray square), 1*s*-σ* transition of C_{Al}-O (white square), and 1*s*-π* transition of carboxylate (COOR,H; black circle). Note that absorption intensity at and above ~291 eV corresponds to onset of carbon 1*s* absorption edge as well as various virtual states (1*s*-π* transitions) that reside above ionization threshold.

TABLE 1. AVERAGE CHEMICAL STRUCTURES OF RECENT AND FOSSIL TRACHEID WALLS DERIVED FROM C-XANES SPECTRA

Peak	Transition	eV	*Juniperus*		*Metasequoia*		*Asteroxylon*	
			Outer	Inner	Outer	Inner	Outer	Inner
Aromatic carbon C_{Ar}-C,H	$1s$-π^*	285.1	4	4	4.8	4.7	5.1	5.2
Aromatic carbon C_{Ar}-O	$1s$-π^*	287.2	2	2	1.2	1.3	0.9	0.8
Aliphatic carbon CH_{1-3}	$1s$-$\sigma^{*\dagger}$	287.8	—	—	1.4	2.6	0.3	0.7
Carboxylate COOR,H	$1s$-π^*	288.5	—	—	—	0.4	0.5	0.5
Aliphatic ether or alcohol C_{Al}-O	$1s$-$\sigma^{*\dagger}$	289.5	5.4	9.9	3.8	4.7	2.7	3.5
F_A[§]			0.53	0.38	0.53	0.44	0.63	0.56

Note: Carbon chemistry data cast in terms of number of carbons normalized to six aromatic carbons, i.e., a single aromatic ring. — Indicates none detected.

† The low energy $1s$-σ^* transitions involve C-H bonding.

§ Fraction of carbon that is aromatic.

The surprisingly high degree of chemical preservation observed in our fossil samples may be related to the early infiltration of organic material by silica (Boyce et al., 2001), which led to a closed chemical system during subsequent diagenesis. It is particularly encouraging that such a fine degree of chemical preservation is observed in the Rhynie Chert: Rhynie is the source of some of the earliest known terrestrial fungi and arthropods (Kenrick and Crane, 1997), as well as the oldest-known permineralized plants, which play a pivotal role in our understanding of the evolution of vascular plants in general and the tracheid cell type in particular (Friedman and Cook, 2000; Edwards, 1993; Kenrick and Crane, 1991). Analysis of even older permineralized fossils, including microbial assemblages in Proterozoic cherts, may also yield illuminating molecular signatures derived from labile biomolecules. The success of this initial application of scanning transmission X-ray microscopy to the analysis of ancient plants points to a bright future for comparative studies of the biochemistry and physiology of fossil organisms.

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