Isolation and characterization of the earliest taxon-specific organic molecules (Mississippian, Crinoidea)

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ABSTRACT

Biomarkers and other ancient preserved molecules are rapidly being discovered and used to study the evolution of life on Earth. We report the existence of echinoderm-specific organic molecules from different lower Mississippian (340 Ma) crinoid species that occur in the same sedimentary bed. These are the oldest examples of biomarker molecules extracted directly from fossilized remains. These biomarker molecules appear to resemble aromatic or polyaromatic quinones, based upon ultraviolet and visible light spectroscopy, fluorescence excitation-emission matrix spectroscopy, and electrospray ionization time-of-flight mass spectrometry. Results suggest that the preservation of diagnostic organic molecules is much more common that previously realized, and that preserved organic molecules may provide an independent method to unravel phylogenetic relationships among echinoderms and, perhaps, other fossilized organisms.

INTRODUCTION

Stable-isotope signatures and organic molecules indicative of life are known from sedimentary rocks beginning in the Proterozoic (Knoll, 2002), but only rarely have organic molecules been extracted directly from fossils. For example, fragments of DNA from the extinct woolly mammoth are reported from a sample dated at 27,740 ± 220 ^{14}C yr B.P. (Poinar et al., 2006); proteins from the Cretaceous dinosaur *Tyrannosaurus rex* have recently been extracted (Asara et al., 2007; Organ et al., 2008); and a chitin-protein complex has been reported from Paleozoic scorpions (Cody et al., 2011). The endoskeleton of crinoids (phylum Echinodermata) is composed of as many as thousands of individual plates, where each plate is a single crystal of calcite in optical continuity (Donovan, 1991). Paleontologists have recognized for many years that some exceptionally preserved crinoid specimens are fossilized with distinctive color differences, ranging from rich reds, blues, and purples, to earth tones varying from white to very dark gray (Bather, 1892; Laudon and Beane, 1937; Blumer, 1965; Lane, 1973). Whereas color contrast may vary, this phenomenon occurs at many locations and throughout geologic history, even if present only as subtle differences.

During the early 1950s, M. Blumer began work extracting molecules from fossil crinoids with blue and purple colors from Jurassic strata in Switzerland (Blumer, 1951, 1960, 1962a, 1962b; Thomas and Blumer, 1964). Blumer worked on the European Jurassic crinoid *Millericrinus* (now recognized as *Liliocrinus*) and characterized the largest molecules extracted using ultraviolet and visible light (UV-Vis) absorption spectroscopy. He tentatively identified these compounds as polynuclear aromatic hydroxyquinones called fringelites (Hess, 1972). Fringelites are among more than 200 pigments and metabolites known from both living and fossil echinoderms including echinochromes, spinochromes, and gymnochromes (Dimelow, 1958; Blumer, 1965; Stonik and Elyakov, 1988).

Fringelites are a group of molecules that are structurally similar to hypericin compounds extracted from the plant *Hypericum perforatum*, commonly known as St. John's wort (Fox, 1976). The similarities in structure, UV-Vis diffuse reflectance spectra, and their acidity constants (K_a) make it very difficult to distinguish fringelites from hypericin (Falk et al., 1994; Obermüller and Falk, 2001). However, recent work by Wolkenstein (2005) and Wolkenstein et al. (2006, 2008) was able to elucidate fringelites from hypericin using mass spectrometry.

The methods and concepts discussed above, as well as fluorescence excitation-emission matrix (EEM) spectroscopy and mass spectrometry are used here to investigate the presence and identity of molecules preserved in significantly older (Paleozoic) crinoids. Specifically, this study seeks to determine whether the coloration distinction among fossil species results from individual crinoids possessing unique organic molecules, and if so, whether crinoids from a single phylogenetic clade possess molecules that are more structurally similar to one another than to species from more distantly related clades.

MATERIALS AND METHODS

Fossil crinoids were sampled from the lower Mississippian Edwardsville Formation (Morgan and Monroe Counties, southern Indiana, United States; Ausich, 1983). They were chosen in order to test the pervasiveness of biomarkers among fossil crinoids (Fig. 1). The Edwardsville Formation is late Osagean and ca. 340 Ma

Figure 1. Examples of color distinction among different crinoid species on a single bedding surface (Beane Collection, Beloit College, Wisconsin; BC-143), which are distinctive even in grayscale. The species preserved in the lightest color is *Elegantocrinus symmetricus*; two species with darker coloration (different shades) are *Cribanocrinus watersianus* and *Strimplecrinus inornatus*; and one specimen with an intermediate coloration is *Cusacrinus nodobrachiatus*. Magnification ×0.67.

in age. Organic compounds were extracted from crinoids by dissolution in hydrochloric acid followed by centrifugation to pelletize the residual solids. The solid pellet was extracted in a solution of 4:1 vol/vol acetone:methanol, decanted, and extracted again with methanol.

UV-Vis spectroscopy was used to measure the light absorbance of the organics extracted from the molecules. We used this approach to rapidly screen for these and other chromophoric organic molecules (Blumer, 1965; Wolkenstein, 2005). Samples were scanned between 200 nm and 700 nm using a Varian UV-Vis spectrophotometer (Cary® 13) and blanked with the appropriate sample matrix. Fluorescence EEM spectroscopy was used to determine the fluorophoric properties of the extracted organic molecules using the procedures summarized in Cory et al. (2010) and Stedmon and Bro (2008).

All mass-spectrometry assays were performed on a Micromass II electrospray ionization time-of-flight (ESI-TOF) mass spectrometer (Micromass, Wythenshawe, UK) equipped with an orthogonal electrospray source (Z-spray) and operated in positive-ion mode. Sodium iodide was used for mass calibration for a calibration range of molecular mass (*m/z*) of 100–2000. For exact mass measurements, all spectra were combined, smoothed, and centroided; and internal calibration with a lock mass of sodium iodide clusters was used. Optimal ESI conditions were a capillary voltage of 3000 V, a source temperature of 110 °C, and a cone voltage of 55 V. The ESI gas was nitrogen. Data were acquired in continuum mode until acceptable averaged data were obtained.

RESULTS

In our study, the results of Blumer (1965) and Wolkenstein (2005) were duplicated using UV-Vis spectroscopy with the Jurassic crinoid *Liliocrinus* (Blumer and Omenn, 1961). We observed the characteristic hypericinoid peaks (500–600 nm wavelength range) in *Liliocrinus* (Wolkenstein, 2005; Wolkenstein et al., 2006, 2008), but these peaks are absent in the Mississippian species (Fig. 2A) and do not appear to indicate fringelites. Comparisons of the UV-Vis spectra of Mississippian crinoid extracts (Fig. 2A) to known quinones (anthraquinone and purpurin) (Fig. 2B) demonstrate a shared peak at 250 nm, suggesting molecules with a similar structure, but this is not definitive as many aromatic structures absorb at this wavelength. Further, it was demonstrated that the fossils have biomarker molecules distinctive from the encasing rock (O'Malley et al., 2008).

Fluorescence EEM plots in Figures 3A–3H represent emission intensity as a function of excitation wavelength, where the peaks of maximum intensity are characteristic for specific compounds. Fluorescence EEM spectroscopy provides a characteristic "fingerprint" of the fluorophores in a compound (Cory and McKnight, 2005). Therefore, EEM spectra of known compounds can be compared to unknown molecules extracted from fossils. Figures 3F–3H are the EEM spectra for the Mississippian crinoids *Barycrinus rhombiferus*, *Cyathocrinites iowensis,* and *Elegantocrinus hemisphaericus*, and have structures that appear to be quinone- or hydroquinone-like. Thus, these molecules may be the diagenetically altered variants of quinones produced in abundance by living crinoids (Thompson, 1971). Indeed hypericin and fringelites are polynuclear hydroquinones. As a point of comparison, similarities exist between lawsone (a 2-hydroxy-1,4-naphthoquinone) (Fig. 3D) and the fossil extracts. The diagnostic peak for lawsone (Fig. 3D) occurs at an excitation wavelength of 240 nm and emission wavelengths of ~350 and 390 nm. Therefore, we suspect that these molecular fossils contain quinone-like moieties.

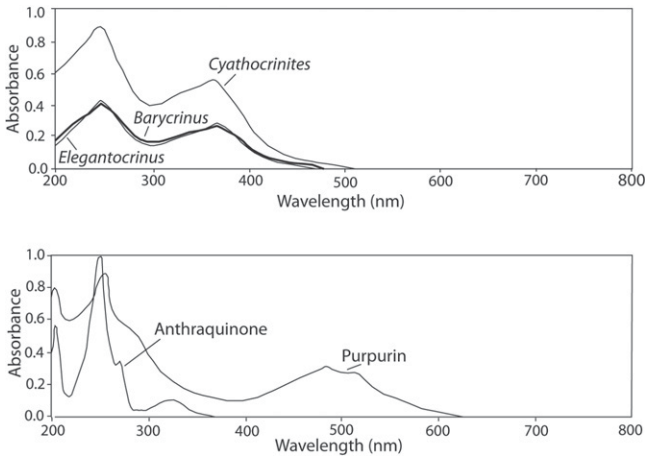

Figure 2. Ultraviolet–visible light spectra for fossil samples and solutions of known compounds. Fossils have peaks at 250 and 380 nm. Anthraquinone has peaks at 205, 255, and 320 nm; purpurin has peaks at 205, 255, and 500 nm.

ESI-TOF mass spectrometry was also used to analyze extracts from several species of Mississippian crinoids. The ESI-TOF mass spectra have peaks representing masses of the molecular-ion peaks (in positive-ion mode) from the extracted organic molecules. Results from three crinoids are presented in Figures 3A–3C. *B. rhombiferus*, *C. iowensis,* and *E. hemisphaericus* demonstrate that each has a unique mass spectrum that is interpreted to be indicative of an intrinsic biological attribute of these organisms when alive. All three crinoids share a common mass of m/*z* 191.02. Two of the crinoids (*Barycrinus* and *Cyathocrinites*) are from the subclass Cladida but belong to different families, Barycrinidae and Cyathocrinidae, respectively. These two crinoids share molecular masses at m/*z* 237.07, 355.06, and 429.08. The molecular mass of m/*z* 355.06 is very close to that of hexahydroxyphenanthroperylene (HHPP, with a m/*z* 358.17) and might be a related derivative (Wolkenstein et al., 2005, 2008). Aside from these common masses, each has a distinct spectral signature that uniquely identifies that taxon. *B. rhombiferus* possesses unique peaks at m/*z* 253.14 and 307.09, whereas *C. iowensis* has molecular masses at m/*z* 186.82, 296.72, 519.05, and 593.07. *Elegantocrinus* is from the subclass Camerata and is clearly distinct from the two cladid crinoids with mass peaks at m/*z* 159.00, 219.02, 307.02, 508.92, 537.88, and 569.90.

The lower molecular masses of these molecules (*m/z* 190–260) suggest that they may be smaller polynuclear quinones; e.g., naphtho- or anthraquinones. The most abundant molecular species has a mass of m/*z* 237.07, which is similar to the molecular weights of anthraquinones present in modern crinoids (Thompson, 1971). Because these data were collected from a complex mixture and the components were not separated by high-performance liquid chromatography (HPLC) prior to ESI-TOF mass spectrometry analysis, we cannot use these results to determine its exact composition. However, we have determined that there are peaks that exist in common (green) and peaks that are different (purple, orange, yellow; Figs. 3A–3C). This indicates that there must be similarities and differences in the components of each mixture.

The remarkable stability of these compounds results from the physiology of echinoderms. We recognize three factors as primarily responsible for the preservation of taxon-specific organic molecules from Mississippian and Jurassic crinoids. First, as mentioned above, these crinoids were buried rapidly in fine-grained sediments that preserved the fossils well and isolated them from significant exposure to fluids moving through the enclosing rocks that could leach or otherwise alter the organic and inorganic composition of these fossils. Second, the microstructure and crystallinity of crinoids and other echinoderms are unique. The crinoid skeleton is formed within mesodermal tissue. When alive, individual crinoid ossicles are a single crystal of the mineral calcite, with ~50% of the volume composed of calcite, and the porosity of the plate is filled with various soft tissues. After death, during early diagenesis, the porosity of individual plates is occluded rapidly by

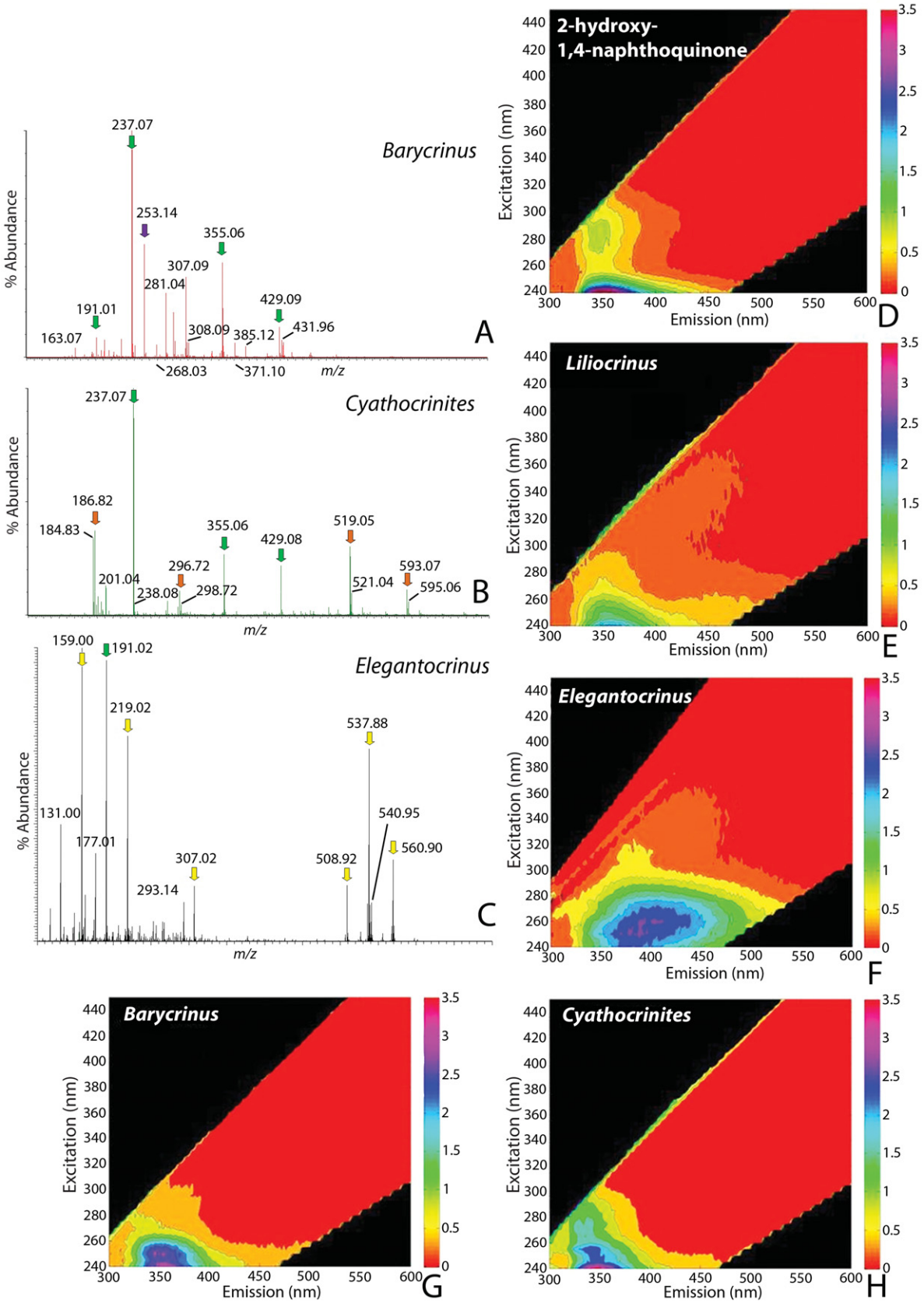

Figure 3. Results from LC (liquid chromatography) time-of-flight (ESI-TOF) mass spectrometry and excitation-emission matrix (EEM) spectroscopy. A–C: ESI-TOF mass spectrometry results with common peaks indicated by green arrows and taxon-specific peaks indicated by purple, orange, and yellow arrows (molecules in positive-ion mode); peaks are labeled with molecular mass (*m/z*). A: *Barycrinus rhombiferus*. B: *Cyathocrinites iowensis*. C: *Elegantocrinus hemisphaericus*. D–H: EEM spectroscopy results, in Raman intensity units; samples analyzed in water. D: Lawsone (2-hydroxy-1,4-naphthoquinone). E: *Liliocrinus*. F: *Elegantocrinus hemisphaericus*. G: *Barycrinus rhombiferus*. H: *Cyathocrinites iowensis*.

syntaxial calcite cement that retains the crystallinity of the original ossicle. In so doing, some of the organic molecules from the organism may become trapped within the newly precipitated calcite and preserved (Thomas and Blumer, 1964). Thus, organic molecules from the living organism may be trapped inside a single, relatively large calcite crystal that is geochemically stable over geologic time, unless altered through diagenesis or metamorphism. Dickson (2001) referred to echinoderm ossicles as "crystal caskets", and the trapped organic molecules within a crystal casket are resistant to normal diagenetic change and leaching. The third factor is that the lower Mississippian rocks studied from Indiana have never been exposed to any metamorphism, and they have never been buried deeply, as indicated by a conodont alteration index of 1.5 or less for the deposit under study (Harris et al., 1990; C.B. Rexroad, 2006, personal commun.).

CONCLUSIONS

In conclusion, organic molecules are preserved in and can be extracted from Mississippian crinoid fossils, and they are chemically distinct from country-rock samples. Thus, organic molecules extracted from crinoids must have a biogenic origin and appear to have taxon specificity. Evidence provided by fluorescence EEM spectroscopy and UV-Vis spectroscopy imply that the structures of these molecules are a form of aromatic and/or polyaromatic quinones similar to modern echinoderm pigments. ESI-TOF mass spectrometry results demonstrate that molecules from more closely related taxa are more similar, indicating that a phylogenetic signal is preserved in these ancient echinoderm molecules.

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