Evidence for Endogenous Collagen in Jurassic Crocodilian Bone from the UK

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ANCIENT COLLAGEN ANALYSIS: FROM MICROSCOPES TO MASS SPECTROMETRY

Since the discovery of original biomaterials in ancient bone by Schweitzer in 1993, published reports have grown markedly. Between 1993 and 2019, there were 85 reports of original biomolecules in ancient bone in refereed journals.[1, 2] Today, there are over 150. MS techniques have allowed quantifiable data to be collected, specific proteins to be identified, and correlation of ancient collagen peptide sequences to modern counterparts using protein databases, such as Swissprot.[3] This aids protein decomposition studies, as well as building a picture of how these biomolecules persisted to the present day. However, in all such studies, there remains a need to distinguish endogenous biomaterials from contamination.[4] This is because it was generally assumed that fossilisation destroys all organic components.[5] This study uses a previously successful combination of techniques to identify and sequence collagen from a Jurassic crocodilian bone, providing evidence for endogeneity.

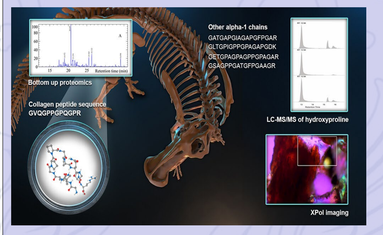


Fig 1. *Recent LC-MS/MS and proteomic results from Edmontosaurus bone, providing evidence for endogeneity.[3]*

These techniques include Fourier-Transform Infrared Spectroscopy (FTIR) to provide evidence of organics within the sample, and an indication for the possible presence of collagen, Liquid Chromatography – Mass Spectrometry (LC-MS) following trypsin digestion to extract and uniquely identify collagen, and LC-MS/MS bottom-up proteomics to compare collagen peptide sequences with previous studies, and those of modern crocodilians. This study provides the first evidence for the oldest original biomolecules so far discovered from fossil bone in the UK, the oldest fossil bone to undergo proteomic analysis.

CROCODILIAN VERTEBRA FROM ORTON PIT, UK

A Jurassic crocodilian bone (CRC: JC001) was excavated from Orton Pit, Peterborough, UK, and is currently held in a private museum collection. The bone identification was confirmed through two independent authorities, a private palaeontological museum in Lincoln, UK, and R. Chandler (Natural History Museum, London, UK).[6] Orton Brick Pit is a historical location for early Jurassic fossils, now closed to any excavations due to the establishment of a Site of Special Scientific Interest in the area, because of high levels of biodiversity.[7] Therefore, good fossil vertebrate bone specimens are almost exclusively in the ownership of museums and galleries.

The pit is an old Victorian clay quarry for brickmaking, and during its early years many significant specimens were excavated, including, amongst many other fauna groups, dinosaurs (Megalosaurus, etc.), marine reptiles (Pliosaurus, Liopleurodon, etc.), and pterosaurs.[8] The fossils are from the Oxford Clay Formation, Peterborough Member, which is an extensive and well-studied deposit throughout southern UK.[9] The prevailing theory is that the Oxford Clay Formation represents a shallow marine environment with minor deposition. The clay build-up forms an anaerobic environment, limiting the decomposition of creatures falling into the ooze, and leading to their eventual preservation. Due to the exceptional preservation of specimens within the Oxford Clay Formation, it has become synonymous with dinosaurian research since the Victorian period. There have been two genera of crocodilians excavated from Orton Pit, *Steneosaurus* and *Metriorhynchus*, with this project's vertebra most likely belonging to the latter genera, based off size and morphology.[10]

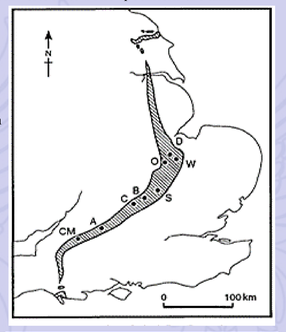


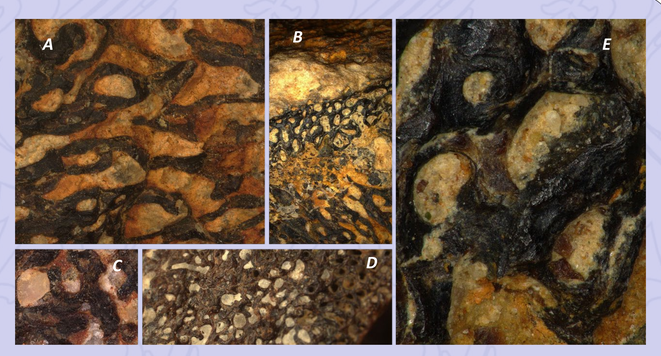
Fig 2. *Oxford Clay Formation in the UK, with significant exposures marked. 'O' is Orton Pit. From Hudson & Martill, 1994. [10]*

SAMPLE PREPARATION

A 1.5-gram sample was taken from the centre of the vertebra, using a rotary drill. The equipment was fully sterilised using ethanol before use, and the sample was taken in a laboratory environment to minimize the chance of contamination. The first 3 mm of the core was discarded to avoid surface contamination, and the equipment was re- sterilised before the sample was taken. This ensured that the sample represented the actual preserved remains of the vertebra. The sample was ground into a fine powder (<50 microns), using sterilised instruments. The ground sample was transferred to a sealed sample aliquot and kept at a low temperature (< 18 °C) until use.

Fig 3. *Crocodilian vertebra showing surface and internal detail.*

Fig 4. *Optical analysis of the crocodilian bone using Keyence VHX digital microscope. The vertebra has distinct differentiation between the bone material and minerals (A, B), while the minerals themselves are well-developed and crystalline (C). This is indictive of strong permineralization, where the minerals have been in the right condition to develop to their full extent, entrapping the bone material. Using Keyence software, we also were able to measure the approximate percentage of permineralization on a 2-dimensional scale. The bone measured on average at 31% ratio of mineral to bone (D). The imaging also gave an opportunity to analyse aspects of bone structure, particularly differentiating between cortical and trabecular structures (E). Given the denser nature of cortical bone, there may be more chance of original biomaterials being preserved inside, where the minerals have not fully permeated the bone structure itself, but have encapsulated the bone.*



LC-MS ANALYSIS IN ANCIENT BONE

Previous analysis results indicating collagen presence

LC-MS is considered the gold standard for collagen detection and analysis. Previous LC-MS tests performed on modern bovine collagen, modern turkey bone dating from 2022 with high collagen content, well-preserved *Palaeotherium* jawbone (2023) and *Iguanodon* vertebra (2024) has been previously published at ASMS.[11, 12] Comparison of LC/MS results showed time stamp peak correlation between the control bone samples (bovine & turkey) and the *Iguanodon/Palaeotherium* bone. Results also showed correlation with the wild boar sample, with 6 – 8 peaks in the mass range of 1082 – 1086 occurring at the same m/z value as those in the modern bone. Relative heights of the corresponding peaks from all samples (bovine, turkey, *Iguanodon*, and *Palaeotherium*) are also in a similar ratio, howbeit at a much-reduced signal intensity. The MS peaks from the modern wild boar *Palaeotherium*, and *Iguanodon* bone correspond with peptides consistent with type 1 collagen (see fig.5). The differing retention times between 2 upper and 2 lower traces are almost certainly due to differing LC column lengths (100mm in upper case and 50 mm in lower case). The masses however shown on the second figure show good agreement which strongly indicates collagen presence in all the bone samples. As such, there was indication that proteomic analysis would be feasible in these, and other, ancient bone samples.

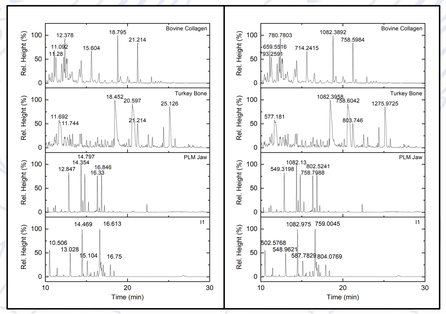
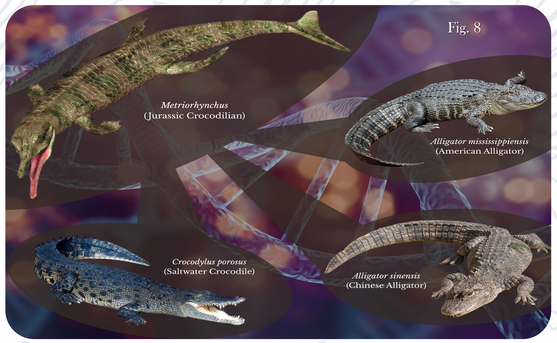


Fig 5. *LC-MS/MS results bovine collagen, turkey bone, Palaeotherium jaw (PLM_Jaw), and Iguanodon vertebra (I1). Left figure shows retention times, right figure shows relative masses.*



PROTEOMIC ANALYSIS

Analysis of the LC-MS/MS data was by bottom-up proteomics and initially performed using Peaks Studio 10.6. Data were searched against the Swissprot database, and then later against the UniProt TrEMBL Birds and Reptile data base, which specialises in unreviewed proteins (i.e. proteins not yet seen in experiments), based on genome data.[13] The search parameters included fixed carbamidomethyl modification of cysteine and variable oxidation of methionine, a precursor mass tolerance of 10 ppm, a product mass tolerance of 0.01 Da, and a maximum of one missed cleavage. As collagen had previously been identified in most of the samples using LC-MS/MS, the data results were filtered for collagen proteins. In the Jurassic crocodilian vertebra, a total of 37 collagen proteins were identified from 437 peptide sequences. Three of the protein matches are collagens specific to crocodilians, matching with the saltwater crocodile (*Crocodylus porosus*), the American alligator (*Alligator mississippiensis*), the Chinese alligator (*Alligator sinensis*) (See Fig. 6 & 8). Other matches included the leopard gecko (*Eublepharis macularius*). The same collagen protein (Collagen Alpha 2) was also identified using UniProt TrEMBL as was identified in Swissprot. The results from the crocodilian bone were also compared to those from an ancient Roman butchered pig bone (c. 100 AD), and an ancient camelid bone, both with known collagen. The results showed peaks for collagen along similar time stamps, albeit at reduced intensities for the crocodilian bone (See Fig. 7). The positive detection, identification, and sequencing of specific collagen types is significant to the question of sedimentological conditions in relation to the preservation and presence of endogenous biomolecules within ancient bone.[3] The endogeneity of the sequenced collagen is supported by not only like-for-like matches in the fossil genera, but also matches to the relevant clade, in the case of the crocodilian bone. With the crocodilian bone, we have the benefit of the same order existing today, relatively unchanged since the existence of the fossil bone.[14] Given the lack of crocodilian contamination potential at the excavation location for the bone (Peterborough, UK), and the steps taken to avoid any form of contamination during the research process, the chances of specific crocodilian contamination in the sample are low. The positive identification of crocodilian collagen peptides within the fossil bone strongly suggests the extracted and sequenced collagen is endogenous.

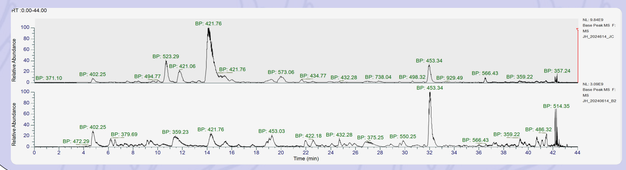


Fig 7. *LC-MS results for the Jurassic crocodilian bone (top) and ancient camelid bone (bottom), with the basepeak labelled, showing similar timestamp peaks for collagen. Note that the BP 421.76 is the Trypsin Autolysis peak used in the bone digestion process for analysis.*

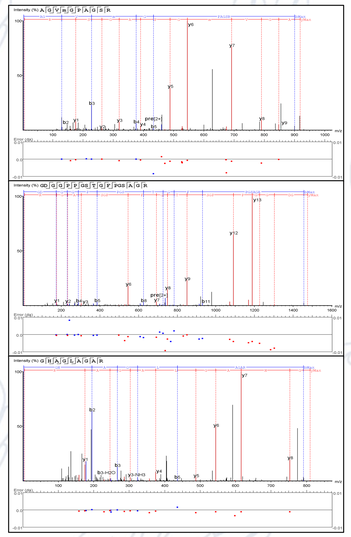


Fig 6. *LC-MS MS2 spectra for the three crocodilian peptides. Spectra records a series of b ions and y ions, with the m/z value differences indicating the amino acid sequence and identifying the peptide.*

CONCLUSIONS

Collagen is a principal component of animal and human bone, being the main structural protein in extracellular matrix.[15] The presence of collagen in ancient bone (particularly fossil bone) is of current interest and has been both claimed and contested. [16, 17] This study provides additional evidence from LC-MS/MS (following protein digestion) of the presence of Type 1 collagen in our bone samples. Bottom-up proteomics provide conclusive evidence of the collagen identified being endogenous, with specific peptides matching those of modern crocodilians. These results make this the geologically oldest experimental observation of original biomolecules within fossil bone from the UK. The additional sedimentological data allows for comparison with other results from ancient (fossil) bone from different geological sequences from the Jurassic to Holocene.

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