# First histochemical examination of a Miocene ostrich eggshell with the oldest mineral-bound peptides postprint

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## Abstract

Ancient proteins possess higher preservation potential than ancient DNA, and thus proteomic research can help elucidate biological characteristics of extinct organism groups that lie beyond the scope of ancient DNA studies. The oldest peptide discovered to date was found in ostrich eggshell fossils from the Late Miocene Linxia Basin in northwestern China, representing a portion of the eggshell mineralization-related protein struthiocalcin (SCA-1). Previous researchers believed that SCA-1 was uniformly distributed throughout the eggshell and could be preserved over geological time scales due to its capacity to bind with calcite crystals. The present study conducted histological, scanning electron microscopy, and Raman spectroscopy analyses on the same ostrich eggshell fossil, revealing that the crystal nuclei within the inner cone layer of the eggshell contained partial apatite, while other regions were composed entirely of calcite; these nuclei regions must have undergone phosphatization during diagenesis. Following decalcification treatment of the fossil eggshell sample, residues were observed in the cone layer nuclei regions, exhibiting a network-like fibrous structure whose location and morphology resemble those of organic matter residues after decalcification in modern ostrich eggshells. These results indicate that ancient peptides in this fossil eggshell may be concentrated and preserved at the cone layer nuclei rather than being uniformly distributed throughout the entire eggshell. Phosphatization may represent an additional taphonomic process conducive to the long-term preservation of organic matter. The paleoclimate and burial environment of the Linxia Basin may have provided favorable conditions for the preservation of this ancient protein molecule. It is recommended that future research conduct more comprehensive histochemical and mineralogical analyses to further elucidate the preservation mechanisms of organic matter and ancient proteins in this basin.

# Full Text

## Preamble

### First Histochemical Examination of a Miocene Ostrich Eggshell with the Oldest Mineral-Bound Peptides

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**Abstract:** Ancient proteins have higher preservation potential than ancient DNA, enabling proteomic studies to illuminate the biology of extinct groups beyond the reach of ancient DNA research. The oldest peptide discovered to date is part of struthiocalcin (SCA-1), a protein involved in eggshell mineralization, found within an ostrich egg from the Late Miocene Linxia Basin of Northwest China. It was originally hypothesized that SCA-1 was evenly distributed throughout the eggshell and survived for so long because it was bound to calcite crystals. We conducted histological, scanning electron microscopy, and Raman spectroscopic analyses on this same fossil egg to test whether protein or organic matter could be observed in specific eggshell regions and indeed bound to calcite crystals. Our results show that the eggshell is composed entirely of calcite except at the base layer, which contains mammillary knobs at least partially made of apatite. These knobs were secondarily phosphatized during diagenesis. After decalcification, the fossilized mammillary knobs displayed fibrous residues consistent in location and morphology with remnants of original organic material forming a network, similar to the organic matrix observed in extant ostrich eggshell using the same method. These results suggest that SCA-1 may have been concentrated in the mammillary knobs rather than evenly distributed throughout the eggshell.

Phosphatization may represent another taphonomic process that favors organic preservation over deep time. The paleoclimate and taphonomic environment of the Linxia Basin may have provided favorable conditions for molecular preservation in this egg. More in-depth histochemical and mineralogical analyses will certainly increase our understanding of organic and ancient protein preservation in this basin.

**Key words:** fossil organics, struthiocalcin, apatite, phosphatization, ostrich eggshell, ancient proteins

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## 1. Introduction

The analysis of ancient proteins is an emerging field in paleontology and paleoanthropology that can reveal insights into the physiological, ecological, and evolutionary traits of extinct organisms, including our own ancestors (Hendy et al., 2018; Thomas and Taylor, 2019; Warren, 2019; Hendy, 2021). Among ancient proteins, those involved in biomineralization specifically demonstrate good preservation potential (Demarchi et al., 2016).

Recently, Demarchi et al. (2022) extracted a polypeptide from a fossilized ostrich eggshell found in the Linxia Basin of Northwest China using liquid chromatography with tandem mass spectrometry (LC-MS/MS), representing the oldest ancient peptide sequence discovered to date, dating back to 6–9 Ma (Demarchi et al., 2022). This polypeptide is part of the protein called struthiocalcin SCA-1, homologous to ovocleidin OV-17 of the chick (Arias et al., 2007; Gautron and Nys, 2007; Gautron et al., 2021). Molecular dynamics analysis shows that SCA-1 is tightly bound to calcite particles, making the polypeptide more stable and resistant to degradation (Demarchi et al., 2016). The authors suggested that binding to calcite crystals is one reason why the protein survived over deep time and that the protein was distributed evenly among the eggshell layers (Demarchi et al., 2016, 2022).

Currently, many hypotheses exist about how molecules (e.g., DNA, proteins) are preserved over deep time within tissues. Besides the proposal for SCA-1, researchers think that dry and cold environments slow organic matter degradation and benefit ancient protein preservation (Kendall et al., 2018). Dehydration is believed to favor preservation of non-mineral-bound polypeptide chains (Collins and Riley, 2000). However, without histological analyses, these hypotheses remain speculative. A unification of the fields studying and sequencing ancient biomolecules and paleohistology is necessary to answer these questions. Here, we begin testing pre-existing hypotheses proposed by molecular paleontologists and experts in ancient molecules using this eggshell bearing the oldest known peptide. To test the calcite-bound peptide hypothesis, we analyzed the histology (with ground sections and scanning electron microscopy, SEM) and chemistry (with energy-dispersive X-ray spectroscopy, EDS) of an adjacent eggshell fragment from the same egg. Additionally, we removed all calcite from another fragment

using the decalcifying solution EDTA (ethylenediaminetetraacetic acid) to visualize potential organic matter remaining after calcite dissolution. Overall, the main goal of this study was to improve understanding of peptide preservation in this eggshell and its mode of fossilization.

## 2. Materials and Methods

### 2.1 Material

Two fragments were sampled from the fossil ostrich eggshell (Struthio linxiaensis, IVPP V 26107) from the Late Miocene Liushu Formation of the Linxia Basin in Gansu Province, Northwest China (Demarchi et al., 2022). One fragment was used for petrographic ground-sectioning and SEM-EDS analyses, and another was used for demineralized paraffin sectioning and histological staining. Extant common ostrich (Struthio camelus) eggshell fragments were collected from the IVPP collections for both ground and demineralized paraffin sections for comparison with the fossil material. Due to contrasting material properties, the soft egg membrane of the extant eggshell was removed from the fragment used for ground-sectioning.

### 2.2 Petrographic Ground-Sectioning

Fragments of both fossil and extant eggshell were embedded in EXAKT Technovit 7200 (Norderstedt, Germany) one-component resin, which was cured for 12 hours. The embedded blocks were then mounted onto glass slides. Sections were cut with an EXAKT 300CP accurate circular saw and ground and polished with the EXAKT 400CS grinding system (Norderstedt, Germany) until the desired optical contrast was reached at approximately 30–50  m thickness. Sections were observed under natural and polarized light with a Nikon Eclipse LV100NPOL microscope and photographed with a DS-Fi3 camera and the built-in NIS-Element v4.60 software.

### 2.3 Demineralized Paraffin Sections and Histological Staining

The fragments were embedded in 3% agar (AoBoXing Product 01-023) and demineralized with EDTA (Invitrogen, Thermo Fisher Scientific, 0.5 M, pH 8.0) for 3 days until the eggshell fragment became transparent. The agar blocks were then washed with water to remove any leftover EDTA and subsequently subjected to routine paraffin section protocol (Schweitzer et al., 2016; Bailleul et al., 2020), including dehydration, clearing in xylene, and paraffin infiltration and embedding. Sections were cut at 5  m on a rotary microtome (Leica Biosystems RM2265), placed into a warm water bath (at about 44 °C) with water bath adhesive (Electron Microscopy Sciences Cat.#71303-01) and mounted on charged slides (Superfrost Plus, Fisher Scientific). Slides for SEM and EDS examinations were simply deparaffinized in different xylene solutions for about 15

min. Some other slices were stained with standard alcian blue for the organic matrix of the eggshell following the protocol used in Schweitzer et al. (2016). Stained sections were observed under transmitted and polarized light using the same equipment and software as the ground-section slices.

### 2.4 SEM and EDS

SEM images and energy-dispersive X-ray spectroscopy (EDS) of the ground-section slices and the deparaffinized demineralized paraffin section slices of both fossil and extant samples were taken at the Chinese Academy of Geological Sciences (Beijing) using an FEI Quanta 450 (FEG) at 20 kV without coating. BSE (back-scattered electrons) modes were applied to the slices.

### 2.5 Raman Spectra Analysis

Spot Raman analyses were performed using an Alpha300R Raman spectrometer with a 600 grooves/mm grating and a CCD detector. Data were collected using a laser wavelength of 532 nm and a laser power of 15 mW. The spectra were obtained in the range from 100 to 3900 $cm^{-1}$ at an exposure time of 0.4 s with two data accumulations on the eggshell cross-sections. Acquisition time was 0.4 s per spot.

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## 3. Results

### 3.1 Paleohistology, SEM and EDS on Ground Sections

The fossil eggshell IVPP V 26107 has a thickness of 3.15 mm, significantly greater than that of the extant common ostrich eggshell (2.02 mm). The ground section of the fossil eggshell under transmitted light reveals at least two layers from the inside out: a mammillary layer and a palisade layer [Figure 1: see original paper]. A pore connects the inner and outer surfaces of the shell [Figure 1: see original paper]. The lower quarter of the shell (the mammillary layer) is lighter in color than the upper part, which has a thin dark layer on the inner surface [Figure 1: see original paper]. This thin dark layer corresponds to the location of the mammillary knobs [Figure 1: see original paper]. Under polarized light, the center of the knobs shows an extinction pattern similar to the resin used to embed the samples and quite distinct from the calcite crystals of the eggshell [Figure 1: see original paper]. This is also observed in the extant ostrich eggshell [Figure 1: see original paper].

Backscattered electron (BSE) images of the ground section show that both the fossil and extant eggshells are dense, except for the mammillary knobs which are relatively porous [Figure 2: see original paper]. EDS analysis revealed calcium-rich elements throughout both eggshells [Figure 2: see original paper], consistent with calcite as the major component (Hincke et al., 2012). In the fossil eggshell IVPP V 26107, the pores of the mammillary knob are fewer in number but

larger in size than those of the extant ostrich eggshell [Figure 2: see original paper]. The mammillary knobs of V 26107 are composed of calcium (Ca), some oxygen (O), and are highly enriched in phosphorus (P) [Figure 2: see original paper]. Raman spectra analysis on the mammillary layer of V 26107 showed the presence of both calcite and apatite in the knobs, while only calcite was found in other parts of the mammillary layer.

In the extant ostrich eggshell, the core of the mammillary knob shows higher carbon content and lower calcium content than other parts, with relatively low density indicated by darker color [Figure 2: see original paper]. Although some phosphorus is present in the extant specimen, the concentration is relatively low and evenly distributed without local enrichment [Figure 2: see original paper], unlike what is observed in the fossil. Contrary to IVPP V 26107, Raman analysis did not find any apatite in the extant ostrich eggshell, either in the mammillary layer or the palisade layer, which is consistent with previous studies (Cusack et al., 2003; Yang et al., 2018).

Additionally, some layered material adheres to the mammillary knob of the fossil eggshell [Figure 2: see original paper], which is rich in oxygen (O), aluminum (Al), and silicon (Si) elements [Figure 2: see original paper], indicating it may be clay and silica from sediments, consistent with the burial environment of the Liushu Formation (Deng et al., 2004b). At the bottom of the EDS images of both the fossil and extant eggshell, the embedding resin shows a significant high carbon (C) signal, indicated as bright yellow in the fossil sample [Figure 2: see original paper] and bright red in the extant sample [Figure 2: see original paper].

### 3.2 Paleohistology, SEM and EDS on Demineralized Paraffin Sections

The second fragments of both fossil and extant eggshells were decalcified in EDTA, transformed into 5  m sections, deparaffinized with xylene, and stained with alcian blue [Figure 3: see original paper].

After decalcification, most mineral matrix of both the fossil and extant eggshell dissolved, including calcite and apatite. Some residues with a radial and fan-shaped appearance can be seen at the internal surface of the fossil eggshell [Figure 3: see original paper]. These residues are consistent in morphology, distribution, and size with the mammillary knobs of both the fossil and extant ostrich eggshell [FIGURE:1, FIGURE:3], while all other eggshell layers were completely dissolved in the EDTA solution or did not adhere to the slide [Figure 3: see original paper]. The micro-fibers found in these residues appear connected to each other, forming a network [Figure 3: see original paper]. In the demineralized section of the extant ostrich eggshell, the knobs are connected to the soft egg membrane with a more regular shape [Figure 3: see original paper]. The core of the knobs in the fossil sample appears empty after demineralization [Figure 3: see original paper] and was probably also dissolved in the EDTA solution, just as the other eggshell layers were.

Under SEM, residual calcite crystals (rich in calcium) in the middle of the V 26107 fragment are indicated by bright yellow in [Figure 4: see original paper]. We observed fibrous matter attached to the residual calcite crystals (indicated by white arrows, [Figure 4: see original paper]). The fibrous matter is approximately 42 microns long and 6.5 microns wide at its widest point, tapering at both ends and slightly wider in the middle where it attaches to a calcite crystal [Figure 4: see original paper].

The elemental composition of this fiber is significantly different from that of the surrounding calcite, with very little calcium but rich in carbon, suggesting it may be a remnant of original organic material that was slightly calcified.

### 3.3 Raman Spectra Analysis on Ground Sections

Raman spectra of the mammillary knob on ground sections of both fossil and extant ostrich eggshell show obvious bands at 283 $cm^{-1}$ and 1085 $cm^{-1}$, corresponding to the calcite vibrational pattern and consistent with calcite as the main mineral in eggshells [Figure 5: see original paper].

Raman spectra of the fossil samples also show a clear band at ~961 $cm^{-1}$ [Figure 5: see original paper], which can be attributed to the symmetric stretching vibration of the P-O bond in phosphate radical ($PO_4^{3-}$). This observation, along with results from SEM and EDX analysis [Figure 2: see original paper], indicates that the mammillary knob of the fossil eggshell contains not only calcite but also apatite.

Bands at 1434 $cm^{-1}$ and 2933 $cm^{-1}$ in both fossil and extant eggshell samples may represent some organic matter, which is consistent with bands in the resin used for sample embedding [Figure 3: see original paper], rather than indicating any endogenous organic matter. The resin may penetrate the sample during embedding due to the porous feature of the mammillary knob observed by SEM [Figure 2: see original paper]. Considering the definitive evidence for endogenous organic matter in this fossil and extant specimen [FIGURE:1, FIGURE:3, FIGURE:4] (Demarchi et al., 2022), this implies that Raman spectroscopy may not be effective for detecting organic matter in such resin-embedded samples, or that the endogenous organic matter (protein or peptide) may be too trace to be identified by Raman spectroscopy. This also reminds us to be cautious when testing for organic matter in this type of sample.

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## 4. Discussion

### 4.1 Phosphatization of the Mammillary Knobs of the Fossil Eggshell

EDS and Raman spectroscopy results show apatite concentration specifically at the mammillary knobs of IVPP V 26107 (not in the palisade layer or anywhere else in the eggshell) [FIGURE:2, FIGURE:3, FIGURE:5]. Since no apatite

has ever been reported in the mammillary and palisade layers of extant avian eggshells (also supported by Raman data from this study showing no apatite anywhere in the extant ostrich egg fragment, [Figure 5: see original paper]), and apatite is only found in the cuticle layer of extant avian eggshells (Cusack et al., 2003; Yang et al., 2018), the apatite in V 26107 is diagenetic. The small amount of phosphorus found in the extant ostrich eggshell comes from the organic matrix of the eggshell.

Phosphatization is a relatively common phenomenon during fossilization of soft eggshells, probably through apatite replacement of the membrana testacea (shell membrane). This was observed in the egg of *Hamipterus* from the Early Cretaceous (Li et al., 2022), the egg of *Lufengosaurus* from the Early Jurassic (Stein et al., 2019), and in *Antarcticoolithus* from the Late Cretaceous (Legendre et al., 2020). In extant avian eggshells, the mammillary knobs have significantly higher organic matter content than other parts of the eggshell (Nys et al., 2004; Solomon, 2010; Hincke et al., 2012). Therefore, the phosphatization mechanism involved in the *Struthio linxiaensis* egg studied here may have been similar to soft egg fossilization, where apatite grew onto the template formed by original organic matter at the molecular scale (Zhang et al., 2011; Li et al., 2022).

### 4.2 Preliminary Insights on Organic Preservation

In this study, we found fibrous residues after decalcification from the fossil ostrich eggshell similar in morphology, location, and size to the mammillary knobs of the extant eggshell [FIGURE:3, FIGURE:4]. Since the decalcification solution removed calcium from minerals like calcite and apatite, and since EDS analysis excluded the presence of silica [Figure 2: see original paper], these residues are most likely organic, probably remnants of the original proteinaceous fibrous network found within the mammillary knobs.

A previous study suggested that SCA-1 protein was evenly distributed among the layers and bound to calcite crystals (Demarchi et al., 2016). However, our results suggest a different story. Among mineralized eggshells in extant birds, the mammillary knob is the site of calcification initiation and is rich in organic matter (Nys et al., 2004; Solomon, 2010; Hincke et al., 2012). SCA-1 and its homologous protein OV-17 are not distributed evenly throughout the shell matrix but are concentrated in the mammillary knobs (Hincke et al., 1995; Gautron et al., 2021). The residue found here in the mammillary knobs of the demineralized fossil ostrich eggshell [Figure 3: see original paper] is consistent with the abundance of organic matrix in mammillary knobs and the concentration of SCA-1 in extant avian eggs. Hence, the ancient peptide belonging to SCA-1 found by Demarchi et al. (2022) in the very same egg we re-analyzed here may not be evenly distributed among the layers but instead concentrated within the mammillary knobs.

Additionally, since the mammillary knobs of V 26107 were enriched in phosphorus [Figure 2: see original paper] and contained apatite [Figure 5: see original

paper], our combined results and literature knowledge suggest that phosphatization may also have contributed to the survival of the polypeptide found by Demarchi et al. (2022).

Phosphatization, as an important taphonomic process for fossil preservation, represents the finest taphonomic mode that can preserve subcellular structures (Briggs et al., 1993; Schiffbauer et al., 2014). Some organic molecules such as proteins can interact with apatite crystals and become embedded or adsorbed within the mineral structure, similar to what occurs during bone and teeth formation (e.g., Iline-Vul et al., 2020; Hong et al., 2022). It has been shown experimentally that phosphatization of soft tissues can occur within several weeks (Briggs and Kear, 1993), preventing decomposition of organic matter early during burial and facilitating long-term preservation.

### 4.3 Hypothesized Diagenetic Pathways of the Ostrich Eggshell from the Linxia Basin

During phosphatization, organic tissues such as the egg membrane are a potential source of phosphorus (Schiffbauer et al., 2014). Phosphorus could also come from the environment or decaying organic matter (Orr, 2014), and abundant animal bone fossils found in the strata of the Linxia Basin (Bergmann et al., 2010). As a closed structure, the eggshell can isolate external oxygen and microorganisms, which is conducive to organic matter preservation (McCoy, 2014). During the Late Miocene, uplift of the Tibetan Plateau caused monsoon changes, resulting in arid climates in central Asian regions including the Linxia Basin (Li and Fang, 1999; Deng, 2004a, 2009; Liu and Dong, 2013). A dry environment is also thought to favor preservation of ancient proteins, since breakdown of polypeptide chains into amino acids requires water participation (Demarchi et al., 2016).

Combining microstructural results with EDS and Raman analyses, our study proposes a diagenetic pathway for phosphatization of original organic matter in this ostrich eggshell and other organic preservation in the Linxia Basin [Figure 6: see original paper] (Li et al., 2021).

The process of avian embryonic development leads to resorption of mammillary knobs and separation of the hard eggshell from the shell membrane (Hincke et al., 2018). In V 26107, the mammillary knobs are well preserved, indicating that no embryo ever developed in this egg and that the eggshell including the shell membrane remained intact. Initial decomposition of organic matter in the eggshell may have been prevented by the arid or semiarid environment of the Late Miocene Linxia Basin. After the egg was buried by sediment brought by seasonal precipitation, phosphatization may have occurred quite rapidly, and phosphate minerals may have replaced some organic material within the mammillary knobs. This most likely helped preserve peptides within this eggshell, such as SCA-1, specifically identified by LC-MS/MS in Demarchi et al. (2022). Further work is needed to verify this hypothesis to better understand modes of

organic preservation in this eggshell and throughout the Linxia Basin.

## 5. Conclusions

This study reports the preservation and unique phosphatization process of mammillary knobs in a fossil ostrich eggshell from the Linxia Basin, representing a case of avian eggshell phosphatization during fossilization. We suggest that protein distribution in this fossil eggshell is concentrated within the mammillary knobs, as in extant bird eggshells, rather than homogeneously distributed throughout the eggshell. We propose that phosphatization, perhaps in addition to calcite binding of some proteins, may also play a role in preserving peptides over geological time. This is a preliminary study, and additional in-depth analyses involving protein stains and immunohistochemistry will be necessary to confirm our phosphatization and new apatite-bound peptide hypothesis. We further propose a hypothetical diagenetic pathway for organic preservation in this eggshell and throughout the Linxia Basin. We encourage further analysis of more specimens from the same strata within the Linxia Basin to provide more evidence of molecular preservation and to fully understand mechanisms for organic preservation over deep time.

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*Note: Figure translations are in progress. See original paper for figures.*