Exceptionally preserved extracellular bone matrix proteins from the late Neogene proboscidean *Anancus* (Mammalia: Proboscidea)

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Abstract

In an exceptional preservation state, bones conserve the entire pattern of extracellular bone matrix proteins over thousands or sometimes even millions of years. Here we present typical extracellular bone matrix proteins, which were extracted from a 3.0-million-year-old gomphothere proboscidean, and identified with special antibodies. For the first time, osteonectin, osteopontin and BMP-2 were confidently identified from the extinct *Anancus arvernensis*, based on late Pliocene material from Willershausen, Lower Saxony, Germany. Our study has value in demonstrating that the longevity of original extracellular bone matrix proteins is much greater than formerly expected, and that such materials may be stabilised for distinct geological periods of time, especially in Fossil Lagerstätten.

Keywords  Proteomics · Extracellular bone matrix proteins · Proboscidea · Pliocene · Konservat Lagerstätte · Willershausen · Germany

Introduction

Biological molecules have varying stabilities and preservation potential over extended periods which depending on where these molecules performed their metabolic function during lifetime (e.g., overviews in Demarchi et al. 2016; Ehrlich et al. 2013; Thomas and Taylor 2019; Wysokowski et al. 2014). Molecules, which were synthesized by osteoblasts and secreted to the extracellular matrix (ECM), build the organic part of the bone matrix, an array of proteins collectively termed osteoid. ECM of bone contains only 10% water, whereas cells have a water content of more than 70%. ECM-proteins are embedded and bounded to the calcified structures (e.g., calcium phosphates, comparable with hydroxyapatite) and to collagen. Immediately after the cell death, the molecules inside a cell start rapid degeneration (Morgan et al. 2008). Therefore, the chance to survive over a long time span after death is much better for ECM-proteins than for proteins inside of cells.

The present study deals with the analysis of typical extracellular bone matrix proteins taken from cortical bone of the femur of a late Pliocene gomphothere proboscidean (*Anancus arvernensis*; Elephantidae; Klähn 1932; Göhlich 2012; Karl et al. 2013). The exceptional preservation of these proteins (cf. also Buckley and Wadsworth 2014; Wadsworth and...
Buckley 2014) and other biomolecules and chemical compounds (e.g., Keely et al. 1994; Manning et al. 2019; Volk- enstein and Arp 2021) was caused by special taphonomic features, well known from the limnic Konservat Lagerstätte Willershausen (e.g., Briggs et al. 1998; Briggs 1999).

Materials and methods

A small (ca. 6 × 8 mm) and a large (ca. 18 × 8 mm) sample were taken for microscopic and biochemical (proteomic) analyses from the cortex of the neck of the left femur (GZG.W.35028) of the approximately 3.0-million-year-old extinct proboscidean (Anancus arvernensis) from Willershausen, western foreland of the Harz Mts., which dates from the late Neogene time (Pliocene: Piacenzian; e.g., Wegele 1914; Brauckmann and Gröning 2002).

In the former clay pit of Willershausen, dark laminated clays containing a single carbonate horizon were exposed. These sediments were deposited in a small meromictic lake with anoxic, hyaline bottom water (Meischner and Paul 1982; Meischner 1995, 2000). The great majority of Willershausen fossils have been excavated from the carbonatic sediments (e.g., Krüger 1979; Meischner and Paul 1982; Meischner 1995, 2000; Dlussky et al. 2011; Kolibáč et al. 2016). This site is well known for exceptionally preserved fossils, including cuticles (e.g., Straus 1969; Briggs et al. 1998; Knobloch 1998) and soft parts (e.g., Westphal 1967; Straus 1969; Rietschel and Storch 1974; Špinar 1980; Gehler and Reich 2012; Wolkenstein and Arp 2021). However, the material used in our study was found in the clay during a fossil-digging campaign in the early 1930s, and is part of a partial skeleton of Anancus arvernensis (Fig. 1; see Klähn 1932; Reich and Gehler 2011; deposited at the Geoscience Centre of the Georg-August University of Göttingen, GZG).

From each sample, two thin-ground sections were prepared according to the procedure described by Schultz and Brandt (Schultz 1988, 2001; Schultz and Drommer 1983). These thin-ground sections were established as cross sections at right angles to the longitudinal axis of the femur neck to investigate the morphological integrity, as well as the diagenetic overprint and the degree of fossilization, to rule out possible contamination (Schultz 1997). The thin-ground sections with a thickness of 20–40 µm were studied through the microscope (Leica DM-RXP/DFC 500) in plain and polarized transmission light using a hilfsobject red first order (quartz) as compensator (λ-plate).

The ECM-proteins were extracted as previously described (Schmidt-Schultz and Schultz 2004, 2005; Schultz et al. 2007). The proteins were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (Laemmli 1970) and stained with silver (Swain and Ross 1995).

The 2-D-electrophoresis separates proteins by their isoelectrical point (IPG-strips 7 cm pH 3–10, BioRad USA). IPGs are transferred to the SDS-Page without stacking gel and separated by their molecular weight and also stained with silver. This procedure represents an overview about the quantity of ECM-proteins in the proboscidean bone.

In Western Blot, proteins were identified using special antibodies. In this case, BMP-2 (1:400, Santa Cruz, Inc., USA), osteopontin (1:1000, Calbiochem, CA, USA) and osteonectin (1:1000, Chemicon, CA, USA) were investigated. Horseradish peroxidase (HRP)-linked anti-goat (1:5000, DAKO, Denmark) and HRP-anti rabbit (1:100.000,

Fig. 1 Partial semi-articulated skeleton of Anancus arvernensis, excavated from the Pliocene (Piacenzian) clay of Willershausen by Hermann Schmidt in 1930 (sitting here in the picture; GZG archive), former curator of the Göt- tingen geological collections. The investigated (incomplete) left femur (GZG.W.3502b) is indicated by a red arrow.
DIANOVA, Hamburg, Germany) were used as secondary antibodies. Bands were visualized using enhanced chemiluminescence (ECL)-Plus detection system (Health Care, Freiburg, Germany).

Results

No obvious signs of a strong fossilization process and only minor vestiges of diagenetic alterations could be observed in the bone matrix of the investigated samples (Fig. 2). As the light microscopic investigation showed, the external zone of the bone, containing the external circumferential lamellae, the external tangential lamellae and some blood vessel canals (e.g., Haversian canals) of the cortex, is exceptionally well preserved (Figs. 3, 4). The blasting of external thin bone layer (Fig. 4) was apparently caused by the tension formed during the million-year-long storage in the ground and built up as a result of lithification and load of sediment. At the first view, the larger internal zone including the endosteal zone is not as well preserved as the external (Figs. 3, 5). These areas show in the plain, as well as in the polarized transmission light, a cloudy dark appearance (Figs. 3, 5a, b, 6, 7) which might be due to the original embedding of the Anancus skeleton in dark clays, deposited under anoxic bottom conditions in the center of the former Willershausen pond. If the transmission light is significantly intensified, i.e., overexposed, well organized, regular collagen fibers become visible in the polarized light, which are ordered and expressed in the same way as the fibers in the external zone (Figs. 5c, 7c, d). The presence of these well-developed fibers shows that the cloudy, dark appearance of

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Fig. 2 Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (small sample) of Willershausen Anancus arvernensis viewed through the microscope in plain (a) and polarized transmission light using a red first order (quartz) hilfobject as compensator (b). The external bone surface has been slightly eroded postmortem during embedding and diagenesis. As a result, larger bone particles and smallest bone fragments burst and partially filled secondary external vascular channels. **Scale bar** 160 µm

Fig. 3 Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (large sample) of Willershausen Anancus arvernensis viewed through the microscope in plain (a) and polarized transmission light using a red first order (quartz) hilfobject as compensator (b). The bone tissue in the external area is particularly well preserved. The magenta-colored frames mark the position of the cutout magnifications: 1 = Fig. 4; 2 = Fig. 5; 3 = Fig. 6; 4 = Fig. 7. **Scale bar** 1 mm

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canals (e.g., Haversian canals) of the cortex, is exceptionally well preserved (Figs. 3, 4). The blasting of external thin bone layer (Fig. 4) was apparently caused by the tension

the bony tissue (Figs. 3, 5a, b, 6, 7a) was not caused by the diagenetic action of micro-organisms and that the original bone substance including its matrix is also well preserved
in these dark areas. Thus, the bony tissue was secondarily pigmented by the dark embedding sediment. This result is extremely important and an absolute prerequisite for further biochemical, i.e., proteomic analysis.

The extracted proteins, which represent typical ECMs, were resolved by SDS-Page according to their molecular weight (Fig. 8a). A clear pattern of about 25 different colored bands, stained with silver, in the range of about 10–250 kDa could be detected. In the 2-D electrophoresis more than 300 different protein spots were stained with silver (Fig. 8b). Even if we consider that several of the extracted matrix proteins represent isoenzymes or proteins with different glycosylation groups, we are sure that most ECM-proteins can also be solubilized from a 3.0-million-year-old fossil bone.

Also in western blot we checked for osteopontin, osteonectin and BMP-2. It was possible to identify all three ECMs with their correct molecular weight: osteopontin 45 kDa, osteonectin 40 kDa and BMP-2 28 kDa (Fig. 8c).

Fig. 4 Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (large sample) of Willershausen Anancus arvernensis viewed through the microscope in plain (a) and polarized transmission light using a red first order (quartz) hilfsobject as compensator (b). The external area is particularly well preserved. 1 = Splintered external layer of the cortex; 2 = Sediment fillings above and below the chipped lamella, formed after embedding, during lithification; 3 = Medium, at first glance poorly preserved area of the cortex. Scale bar 160 µm

Fig. 5 Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (large sample) of Willershausen Anancus arvernensis viewed through the microscope in plain (a) and polarized transmission light using a red first order (quartz) hilfsobject as compensator (b) and (c). The endostal zone of the cortex does not seem to be as good as the outer zone preserved (a and b); in the overexposure, however, the collagen fibers present themselves very well and prove a comparatively good condition (c). Scale bar 160 µm

The antibody reactions were controlled by their special blocking peptides.
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**Discussion**

The result of the microscopic examination in plain transmission light gives the impression at first glance that the embedding of bone tissue in a former meromictic pond is not conducive to the preservational status of the collagen (cf. Schultz 1997) and other ECMs (Figs. 3, 5a, b, 6, 7a). However, the collagen fibers and apparently the other proteins of the extracellular bone matrix are not affected in their state of preservation, as shown by our proteomic analysis. This finding is supported by the study of thin-ground sections in polarized transmission light, in which the collagen fibers present themselves as well preserved in case of overexposure (Figs. 5c, 7c, d). It can therefore be assumed that the embedding of bones in a limnic Konservat Lagerstätte also preserves the extracellular bone matrix and does not damage its preservation. The phenomenon of the cloudy dark appearance in the microscopic examination of the thin-ground sections, which is apparently due to the original embedding of the Anancus skeleton in dark clays, can

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**Fig. 6** Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (large sample) of Willershausen *Anancus arvernensis* viewed through the microscope in plain transmission light. The bone structure located in the middle area of the cortex shows no details in the plain transmission light. *Scale bar 160 µm*

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**Fig. 7** Thin-ground section prepared with a thickness of 20 µm from the cortex of the neck of the left femur (large sample) of Willershausen *Anancus arvernensis* viewed through the microscope in plain (a) and (b) and polarized transmission light using a red first order (quartz) hilfsobject as compensator (c) and (d); b and c overexposed. In the overexposure, the collagen fibers present themselves very well and prove a comparatively good condition. In the overexposure it can be seen that the collagen fibers are well to very well preserved. *Scale bars 160 µm (a–c), 64 µm (d)*
possibly be associated with the Maillard reaction products (Wiemann et al. 2018).

BMP-2 has pleiotrophic functions that range from skeletal and extra skeletal organ genesis to generation and regeneration of bone and, additionally, recapitulates the process of embryonic and enchondral ossification and induces bone formation in postfetal life. Thus, BMPs are members of a class of ancient highly conserved signaling molecules that play major roles in embryonic axis determination, organ development, and tissue repair through the animal kingdom (Kaplan and Shore 1998). Osteopontin has a Gly-Arg-Gly-Asp-Ser (GRGDS) amino acid sequence that promotes osteoclast attachment via cellular integrin αvβ3 (Reinholt et al. 1990; Heinegard 1995). The expression of osteonectin (SPARC) is increased in tissues undergoing repair or remodeling due to wound healing, however, particularly in pathological processes, such as formation of cancer metastases, arthritis, diabetes or kidney (Reed and Sage 1996). Up to now, osteonectin, osteopontin and BMP-2 were not identified in a 3-million-year-old proboscidean bone.

Since the late 1980s, several articles were published dealing with the topic ECM-proteins in mastodon bones (Tuross 1989; Schaedler et al. 1992; Asara et al. 2007). In a South American, about 11,000–13,000-year-old radiocarbon dated mastodon skeleton, Schaedler and co-workers microscopically looked for collagen fibers. They compared in gel electrophoresis CNBr peptides of the demineralized bone matrix, obtained from the mastodon (Mammutidae), with the results of mammoth (Elephantidae), modern elephant and rat tail tendon (Schaedler et al. 1992). In the bone of Taima-Taima mastodon, Tuross (1989) looked for the pattern of amino acids, however, as a drawback of this technique, she could not obtain information about the completely preserved matrix proteins (Tuross 1989). In gel electrophoresis, Tuross (1989) detected three different protein bands, for one band she supposed the presence of albumin because of the similar molecular weight. Other related investigations on Pleistocene mammals were published recently (e.g., Cappellini et al. 2012; Hill et al. 2015; Kostyukevich et al. 2018).

Particularly Schweitzer and her group (Schweitzer et al. 2013), who was very active and successful during the last 15 years, sequenced protein fragments from bone collagen of a 100,000 to 600,000-year-old mastodon and a 68-million-year-old dinosaur (Asara et al. 2007). However, also other groups identified non-collagenous ECM proteins such as osteonectin and osteopontin in 120 ky Pleistocene mammals using mass spectrometry (Cappellini et al. 2012; Hill et al. 2015; Kostyukevich et al. 2018), while Kostyukevich and colleagues also detected with coupled liquid chromatography lipids (Kostyukevich et al. 2018). Also impressive is the work of Cappellini and his group dealing with tandem mass spectrometry which enables the sequencing of collagen type I from a 1.5-million-year-old sample of a tooth from Cretaceous fossil remains (Cappellini et al. 2019). The presence of osteocytes and endogenous molecules found by this investigation is exciting (Cleland et al. 2012). Although the perspective is encouraging for future biochemical research on ancient bones, it is unlikely that one will be able to isolate and identify intact cellular proteins, since after death the cell

Fig. 8 Results of the biochemical analyses: a 1-D-electrophoresis (11% T, 2.5% C) ECM-proteins stained with silver. Left lane: molecular weight marker. Right lane: ECM-proteins of the Willershausen Anancus arvernensis femur. b 2-D-electrophoresis of ECM-proteins of the Anancus femur stained with silver. First dimension IPG-strip pH 3–10, second dimension SDS-PAGE (11% T, 2.5% C), on the left, mass marker. c Identification of Anancus ECMs using specific antibodies. Band 1: bone morphogenetic protein–2 (BMP-2). Band 2: osteonectin. Band 3: osteopontin. On the right: molecular weight marker
proteins, along with the decay of the cells, are also broken down very quickly.

Our ECM-proteins extraction protocol differs from most other protocols. After demineralization and centrifugation, most researchers suppose that all the proteins are solubilized in the supernatant after this procedure. Therefore, they use in the further investigation only the supernatants. Cleeland and co-workers as well as Schweitzer and co-workers compared, discussed and summed up the various extraction protocols (Cleeland et al. 2012; Schweitzer et al. 2013). We have found that, after the demineralization process, only a few ECM proteins are solubilized in the supernatant, however, most proteins apparently are still bound very tightly to the residuals of minerals and collagens in the pellet (cf. also Demarchi et al. 2016). We do not use the supernatants for further investigations because protein fragments have high affinity to the bone matrix, which of course also applies to the broken down proteins from other organs, protein fragments from previous investigators, and protein fragments of contaminants from outside, these proteins can be detached more easily and are then in the supernatant. Thus, we solubilized the proteins from the partly demineralized and hence dispersed pellet (Schmidt-Schultz and Schultz 2004, 2005; Schultz et al. 2007). This methodical approach enables researchers to identify various typical bone matrix proteins and also proteins which can support the diagnosis of pathological conditions in dry bone (Schultz et al. 2007).

Conclusions

In our study, we have clearly shown that original extracellular bone matrix proteins, despite slightly diagenetic alteration, can be extracted from bones ~ 3 million years old. Given the success in the late Pliocene Anancus arvernensis material from Willershausen, a limnic Konservat Lagerstätte, the potential for further biochemical (proteomic) studies on older bone material from Paleogene or Mesozoic Fossil Lagerstätten, that can be obtained using specific extraction methods, is evident.

Additionally, using microscopic techniques, micro-morphological changes and characteristics in bones that are of interest to check the diagenetic alteration and to reconstruct taphonomic pathways and the former palaeoenvironment, were presented.

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