Protein Preservation in Fossil Whale Bones of the Miocene/Pliocene Pisco Formation, Peru

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Loma Linda University

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Protein Preservation in Fossil Whale Bones of the Miocene/Pliocene Pisco Formation, Peru

by

Uriel Leme Vidal

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

December 2010
Each person whose signature appears below certifies that this thesis in his/her opinion is adequate, in scope and quality, as a thesis for the degree Master of Science.

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ACKNOWLEDGEMENTS

I thank God for bringing me here thus far, for giving me strength to continue, and for allowing me to participate in His work. Although I am unworthy, He still blessed me abundantly through the course of this study. May this be useful to His work as He sees fit.

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A special thanks to my family, without whom I could not make it. All their prayers and support made up for my lack in areas that I needed to grow and continue to grow.
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<tr>
<td>ABC</td>
<td>Ammonium bicarbonate</td>
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<td>Gla</td>
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<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
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HAc    Glacial acetic acid
HCCA   α-Cyano-4-hydroxycinnamic acid
HCl    Hydrochloric acid
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
L      Liter
LC/MS  Liquid Chromatography-Mass Spectrometry
MALDI-TOF Matrix-assisted Laser Desorption/Ionization-Time of Flight
M      Molar
MeOH   Methanol
Milli-Q water Water deionized by a Millipore Corporation system
ml     Milliliter
μl     Microliter
μg     Microgram
μm     Micrometer
mM     Millimol
MS/MS  Secondary fragmentation
M.W.   Molecular weight
N₂     Nitrogen
NaCl   Sodium chloride
NaHCO₃ Sodium bicarbonate
NaOH   Sodium hydroxyde
nM     Nanomol
P      Pressure unit
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ABSTRACT OF THE THESIS

Protein Preservation in Fossil Whale Bones of the Miocene/Pliocene Pisco Formation, Peru

by

Uriel Leme Vidal

Master of Science, Graduate Program in Biology
Loma Linda University, December 2010
Dr. Leonard Brand, Chairperson

Fossil remains have recently been the focus of considerable attention due to several independent reports of protein and soft-tissue preservation in ancient biological remains. The fossil whales from the Pisco Formation in Peru were previously reported to be very well preserved. A considerable percentage of these whales have their bones still articulated, and not a few have fossil baleen in life position.

In this study we assayed bone samples from some fossil whales for ultrastructure preservation and the presence of original protein/proteinaceous materials. For analysis of fine details of structural preservation we used light microscopy and Scanning Electron Microscopy (SEM). The microscopy results indicated a surprising preservation of both blood vessel and osteocyte shaped structures. For protein/peptide verification we used Micro-Bicinchoninic Acid (BCA) assay for total protein determination, and Fast Performance Liquid Chromatography (FPLC) for proteins/peptides size separation.

The FPLC fractions were analyzed by Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) for a more specific identification of the proteins/peptides sizes. Polyamide sheet Thin-Layer Chromatography was implemented with Dansylated fossil amino acids in order to ascertain the amino acid presence in the
FPLC and the C₈ Solid-phase Extraction (SPE) column elution fractions. Finally, we used Liquid Chromatography-Mass Spectrometry (LC/MS) to obtain the fragmentation pattern of the peptides present in the fossil sample.

The LC/MS fragmentation pattern from fossil material did not show any matching sequence when compared to trypsin digested peptide patterns in the public databases. Nevertheless, it showed secondary fragmentation patterns (MS/MS) characteristic of peptidic material, which implies that it is composed of peptides, but these simply were not cleaved by trypsin, but rather by random (probably unknown) processes. Preliminary DE NOVO sequencing attempts showed that most of the putative peptides in the samples are fragmented, with low molecular weight, but there was also some evidence of higher molecular weight material. Future research is needed in order to determine the protein identification by means of DE NOVO sequencing, and the degree of its preservation in these fossil whales.
CHAPTER ONE
INTRODUCTION

The Pisco Formation

The Neogene Pisco Formation is located in southern Peru, ~350 km south of Lima. It has a desert climate with very little precipitation throughout the whole year. Located near the coast, it became known for its conspicuous abundance of fossil whales. In a study site of approximately two square kilometers, Brand et al. (2004) reported the presence of 346 partial and complete whale skeletons.

Skeletons vary in degree of preservation but most of the whales are still articulated, and a significant number have fossil baleen in life position. This is a particular point of interest because, under current conditions, the baleen detaches from the whale’s mouth in a relatively short time: from a few hours to a few days after death (private communication with marine staff conducting taphonomy research on modern whales).

 Portions of the Pisco Formation are diatom rich, without significant evidence of their dissolution. The high rate of diatom accumulation may imply rapid burial for the fossil whales. Contrary to the generally observed taphonomic processes in recent whale carcasses, little or no evidence of bioerosion is associated with the whales (Brand et al. 2004).
Fossil bone samples brought from the Pisco Formation were analyzed in the Biochemistry and Biology labs at Loma Linda University. Evidence of a high degree of preservation was shown through a variety of tests described below.

**Fossil Preservation History**

The biochemical analysis of what are considered to be ancient fossils is a relatively new field of study. Assumptions of the dominant theory in scientific circles generally excluded most attempts to search for preserved biological macromolecules in fossil remains. Finding them is deemed to be completely unexpected, in view of the immense ages assigned to the fossils.

Notwithstanding the disincentives against unexpected discoveries in this area, the field seems to be thriving with new, scientifically scrutinized, discoveries. There are reports of *ancient* protein all over the world, with special attention to dinosaurian preserved biomaterials, but also from birds, mammals, etc., which are causing a revolution in the common understanding of preservation and protein decay rates.

Abelson (1954) was a pioneer in this paradigm reorganization. He showed that amino acids were found in fossils as old as Jurassic. Weiner et al. (1976) showed that mollusk shells from the Cretaceous still retained identifiable sequences of amino acids that were similar to extant specimens in the same superfamily; protein segments were recovered from a Seismosaurus dinosaur (Gurley et al. 1991); and a variety of accumulating data on the subject has been published in peer-reviewed journals in the last 20 years. But the scientific community only felt the quake when a major breakthrough happened when Schweitzer et al. (2005) reported soft-tissue, vessels and cell-like
structures in *Tyrannosaurus rex*. From then on, this field is gaining more and more attention. In 2007, a new report revealed a partial T-rex amino acid sequence from collagen (Asara et al. 2007).

In order to appreciate at least in part the significance of these discoveries, one must consider the decay, fossilization rates, protein and DNA breakdown rates, that can be estimated by analysis *in vitro* and by some other means (Lindahl 1993; Bada et al. 1999; Poinar 2002), and then, consider the implications of finding remains that still retain their soft-tissue appearance, protein content immunoreactivity, DNA fragments, morphological and some functional characteristics (Hoss et al. 1994; Davis and Briggs 1995; Greenwood et al. 1999; Poinar 1999; Schweitzer et al. 1999a; Jackson et al. 2002; Avci et al. 2005; Asara et al. 2007; Schweitzer et al. 2007b). In view of this, there have been several attempts to propose viable ways by which these ancient biomolecules could have been preserved the way they were (Eglinton et al. 1991; Smejkal and Schweitzer 2007).

Protein preservation seems unlikely to happen through very long ages due to breakdown rates, microbial degradation, diagenesis, and amino acid racemization (Bada et al. 1999; Poinar and Stankiewicz 1999). However, because some fossils seemed to be exceptionally preserved, the idea that original biomolecules could also be present has been suggested in the early 20th century (Moodie 1923).

In later decades, this idea has been strengthened more and more, and now it is well supported (Abelson 1954; Weiner et al. 1976; Westbroek et al. 1979; Armstrong et al. 1983; Collins et al. 1991; Schweitzer et al. 1997a; Schweitzer et al. 1999a; Schweitzer et al. 1999b). For instance, Weiner reported that shells from the late Cretaceous period
yielded repeating amino acid sequences similar to extant shells of the same superfamilies, and that the very labile methionine and cysteine amino acids also occurred in the fossils analyzed (Weiner et al. 1976). Amino acid racemization rates have been suggested, in some reports, as not being always consistent (Weiner et al. 1976; Westbroek et al. 1979). For example: isoleucine is thought to epimerize to alloisoleucine in less than a million years. However, in his samples of late Cretaceous belemnites, Westbroek et al. (1979) found an astonishing absence of alloisoleucine, which he describes as “a most striking feature”.

There is a vast array of researchers who have reported the presence of DNA, polysaccharides, lipids, and protein in fossils from varied locations in the geologic column (Abelson 1954; Shackleford and Wyckoff 1964; Thompson and Creath 1966; Weiner et al. 1976; Westbroek et al. 1979; Hoss et al. 1994; Borja et al. 1997; Schweitzer et al. 1997a; Poinar and Stankiewicz 1999; Schweitzer et al. 1999a; Briggs et al. 2000). Cells and vessels from dinosaurs have been repeatedly reported (Pawlicki et al. 1966; Nowicki et al. 1972; Pawlicki 1975; Pawlicki 1984; Schweitzer et al. 2005).

The antigenic-immunoreactive properties of fossil material have received considerable attention (de Jong et al. 1974; Prager et al. 1980; Collins et al. 1991; Muyzer et al. 1992; Wick et al. 2001), with positive indications that at least parts of the ancient biomolecules are present in original form (Westbroek et al. 1979; Schweitzer et al. 1999a; Schweitzer et al. 1999b; Schweitzer et al. 2002; Schweitzer et al. 2007a). Some went even a further step to show that there can be specific immunological reactions between ancient material and close corresponding animals living today (de Jong et al. 1974; Westbroek et al. 1979). With results like these, research in this field has become
bolder, and it is with much interest that fossils are now being studied at the chemical level.

As for proteins, researchers have reported keratin, albumin, and collagen, osteocalcin, and others as present, at least in part, in ancient remains. Keratin and keratinaceous structures were reported in several fossils such as dinosaurs, mammoths and birds (Gillespie 1970; Norell et al. 1995; Schweitzer et al. 1999a; Schweitzer et al. 1999b). Regarding osteocalcin, which composes about 10-20% of the noncollagenous proteins of bone, Muyzer et al. (1992) reported that at least some preserved immunological properties still exist in upper Cretaceous and upper Jurassic dinosaur specimens, and in Pliocene, Miocene, and Pleistocene mammal and reptile specimens. There was a significantly reduced chance of contamination because immunological results were paralleled with the analysis of a high-quality osteocalcin chemical marker: $\gamma$-carboxyglutamic acid (Gla), which is absent in most microbes and invertebrates (Muyzer et al. 1992), but not all (Low et al. 1980). Albumin has also been identified in ancient biomaterial (Prager et al. 1980; Tuross 1989; Borja et al. 1997).

But of all the proteins found in fossils, collagen has received special attention, and has been identified in a variety of them (Shackleford and Wyckoff 1964; Pawlicki et al. 1966; Towe and Urbanek 1972; Semal and Orban 1995; Schweitzer et al. 1997a; van Klinken 1999; Tuross 2002; Schweitzer et al. 2009). Type I collagen is composed of a triple-helix structure that in bone is composed of two $\alpha_1$ and one $\alpha_2$ polypeptide chains (Weiner and Traub 1992). It is found in connective tissues such as tendons, cartilages, but especially makes up the organic matrix of bone. Collins et al. (1995) conjecture that cross-linked collagen is able to survive more than 100,000 years. The cross-links between
collagen fibrils may be one of the reasons that collagen is relatively more resistant to degradation.

Because water plays a major role in the process of living matter recycling, most preserved fossils are thought to have been deposited in water-free environments (especially in amber, tar pits, and sandy deserts). Some specimens show very little evidence of diagenesis (Schweitzer et al. 1997a; Schweitzer et al. 1997b; Schweitzer et al. 1999b), and should have a higher probability of original preservation. The positive preservation of some kinds of biomolecules (e.g. proteins) increases the chances that other groups of biomolecules (e.g. lipids, carbohydrates, DNA, etc.) might also be preserved in the same sample (Poinar and Stankiewicz 1999; Gugerli et al. 2005; Schweitzer et al. 2007b). The assigned ages to the geologic layers seem to be inconsistent with these discoveries.

The fossil whales from the Miocene-Pliocene Pisco Formation in Peru are ideal subjects for these new approaches to the search for ancient biomolecules. They are very abundant and show a high degree of preservation, with many whales having their skeletons either completely or partly articulated (Brand et al. 2004; Esperante et al. 2007). Some whales are located in places that show an unexpectedly high rate of diatom accumulation, which may have contributed to their outstanding preservation. Therefore, they make good candidates for the analysis that this project is proposing. Previous research on these whales reported in them a creamy matrix and presence of cells after chemical analysis (Schweitzer et al. 2007b). Consequently, we are pursuing further analysis of the biochemical composition of these fossil whales so that important questions can be raised and possibly answered.
Thesis Question

This study will concentrate on answering the following question:

Are there original proteins or proteinaceous material in the fossil whale bones of the Miocene/Pliocene Pisco Formation in Peru?

Goals

i. This research sought to find and identify original proteins or proteinaceous material in the fossil whale bones.

ii. Also, to determine the amino acid sequence of the extracted protein or proteinaceous material.
CHAPTER TWO

PROTEIN PRESERVATION IN FOSSIL WHALE BONES FROM THE
PISCO FORMATION, PERU

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KEYWORDS: Fossil, Cetacean, SEM, Osteocyte, Protein, Pliocene

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Abstract

Fossil whales from the Pisco Formation in Peru were previously reported to show a high degree of structural preservation. A considerable percentage of these whales had their bones still articulated, and not a few still had fossil baleen in life position. In this study we assayed the fossil whales for the presence of original protein/proteinaceous materials. We used the Bicinchoninic Acid assay (BCA assay) for total protein determination, and Fast Performance Liquid Chromatography (FPLC) for proteins/peptides size separation. The fractions were then analyzed by Matrix-assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) for a more specific determination of the proteins/peptides sizes. Finally we used Liquid Chromatography-Mass Spectrometry (LC/MS) in order to determine the identity of the peptides present in the FPLC and the C₈ Solid-phase Extraction (SPE) column elution fractions. The results showed that most of the peptides in the samples are fragmented, with low molecular weight, with some evidence of higher molecular weight material in the sample (~ 5400 Daltons), which could be indicative of better-preserved proteins. Further research is currently being done in order to identify the proteins and their degree of preservation in these fossil whales.
Introduction

Because of the dynamic nature of the Earth’s crust, and the innumerable means by which living matter is constantly recycled, proteins were not commonly expected to be particularly enduring, and little attention has been given to the search for proteins in fossils. The probability of original biomolecules surviving through geologic time is minimal. However, in recent years, research has provided an increasing number of reports of the presence of amino acids/peptides/proteins in a variety of fossils such as dinosaurs (Abelson 1954; Pawlicki et al. 1966; Gurley et al. 1991; Muyzer et al. 1992; Schweitzer et al. 1997a; Schweitzer et al. 1997b; Pawlicki and Nowogrodzka-Zagorska 1998; Embery et al. 2000; Embery et al. 2003; Schweitzer et al. 2009), mammals (Lowenstein 1980; Prager et al. 1980; Lowenstein 1981; Schweitzer et al. 2002), birds (Schweitzer et al. 1999a), fish (Humphreys 1908; Abelson 1954), mollusks (Weiner et al. 1976; Muyzer and Westbroek 1989), and much more, all through the geologic record.

From immunologically responsive original proteins (Westbroek et al. 1979; Huq et al. 1985; Shoshani et al. 1985; Rowley et al. 1986; Muyzer and Westbroek 1989; Collins et al. 1991; Lowenstein et al. 1991; Borja et al. 1997; Schweitzer et al. 1999a; Schweitzer et al. 1999b; Schweitzer et al. 2002; Avci et al. 2005) down through still soft and pliable vessels and tissue (Avci et al. 2005; Schweitzer et al. 2005; Asara et al. 2007; Schweitzer et al. 2007a), the search for original biomolecules in fossils has been receiving increased attention. One of the most reported proteins in fossils is collagen (Isaacs et al. 1963; Ho 1965; Pawlicki et al. 1966; Lowenstein 1980; Franc et al. 1995; Avci et al. 2004; Avci et al. 2005; Schweitzer et al. 2007a), followed by a number of non-collagenous proteins such as osteocalcin, albumin, keratin, etc. The remarkable degrees
of preservation evidenced by some recent discoveries are causing a certain stir in the scientific community (Yeoman 2006).

The current study adds to the growing list, fossil whales from the Pisco Formation. We’ve studied the fossil whales from the desert area of the Neogene Pisco Formation in Peru, which were previously noticed for their remarkable degree of skeletal articulation, and presence, in some specimens, of fossil baleen in original life position (Esperante et al. 2007). In order for such a high preservation to occur, the fossil whales required a very high accumulation rate of diatom and other sediment (Brand et al. 2004).

In this study we assayed the fossil bones at the molecular level in order to better understand its preservation state. We only focused on bone samples, although it is possible that fossil baleen remains may also contain original material. We assayed the fossil bones in order to verify the presence of original proteins or protein fragments. The samples were assayed in order to ascertain if they are made of peptides, and if they can be sequenced.

**Materials and Methods**

Varied fossil whale bones of the Pisco Formation were screened and experimented with. Table 2.1 shows the description of and where we obtained the extant control samples analyzed. Table 2.2 shows the field description and original location of the fossil samples analyzed.
## CONTROLS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Identification</th>
<th>Description and Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken</td>
<td>Femur</td>
<td>Fresh, obtained from a local supermarket</td>
</tr>
<tr>
<td>Cow</td>
<td>Femur</td>
<td>Fresh, obtained from a local supermarket</td>
</tr>
<tr>
<td>SB-Whale</td>
<td>Lower Jaw</td>
<td>20-year after death weathered specimen from Santa Barbara Museum of Natural History, CA.</td>
</tr>
<tr>
<td>Extant Whale</td>
<td>Unknown</td>
<td>Very well preserved. Apparently fresh.</td>
</tr>
</tbody>
</table>

Table 2.1 – Control samples analyzed.

## FOSSILS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Field Identification</th>
<th>GPS Position</th>
<th>Field Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP08-20</td>
<td>Rib</td>
<td>Unknown</td>
<td>Tuffaceous sandstone was stratigraphically a few centimeters below the specimen</td>
</tr>
<tr>
<td>SP08-22</td>
<td>(WIR05-29)</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>SP08-23</td>
<td>Under rib (WIR05-29)</td>
<td>Unknown</td>
<td>Sediment (for control) which was immediately adjacent to bone</td>
</tr>
<tr>
<td>SP08-23</td>
<td>Rib? (WIR05-29)</td>
<td>Unknown</td>
<td>Color is pale and bone seems to be sandy</td>
</tr>
<tr>
<td>SP08-31</td>
<td>Vert. Lat Process</td>
<td>18 436 725 E 839 1311 N</td>
<td>Body was apparently articulated and covered, but skull was exposed and mostly disarticulated. The bone sample itself resembled was mineralized and seemed a very weak candidate for any high degree of preservation.</td>
</tr>
<tr>
<td>SP08-32</td>
<td>Rib</td>
<td>18 437 550 E 839 3919 N</td>
<td>This whale had the baleen still preserved in anatomical position</td>
</tr>
<tr>
<td>SP08-34a</td>
<td>Unknown</td>
<td>18 43 7553 E 839 3921 N</td>
<td>Whale partially disarticulated</td>
</tr>
<tr>
<td>SP08-34b</td>
<td>Unknown</td>
<td>18 43 7553 E 839 3921 N</td>
<td>Whale partially disarticulated</td>
</tr>
<tr>
<td>EFW</td>
<td>Rib</td>
<td>Unknown</td>
<td>Well preserved, with a Buff color, and with sediment completely filling the medullary cavity</td>
</tr>
<tr>
<td>BFW</td>
<td>Rib?</td>
<td>Unknown</td>
<td>Very well preserved, with a color that resembles the purported original white color, common to extant whale bones</td>
</tr>
</tbody>
</table>

Table 2.2 – Fossil samples analyzed (including available field descriptions).
Because of the unusual presence of blood vessel and osteocytes shapes in it, after Ethylenediaminetetraacetic acid (EDTA) demineralization of the bone matrix, the fossil bone sample labeled “BFW” was more extensively analyzed for protein presence. It belonged to a very well preserved fossil baleen whale, cf. Balaenopteridae, which was found at GPS location 18 L 8411328/422633 (datum South American ’69), that is, the upper, diatom-rich, part of the Miocene/Pliocene Pisco Formation in Peru. The field description of the specimen is as follows: “The entire skeleton is articulated and the bones are well preserved, with no evidence of bioerosion or decay of the bone structure. It also has baleen well preserved. The spinal cord has been replaced with black heavy minerals, which are not found anywhere else in the vicinity, indicating that the mineral was deposited in response to the chemical environment caused by soft tissue decay.”

BFW is a rib fragment of this whale. The black heavy minerals were also present toward the center of this bone, and less so in the cortical region. The bone seems to be of a light color, and visually appeared to have relatively less replacement than the other bone specimens, which we also screened.

Protein Extraction

Around 50-55 g of the BFW sample was crushed with a steel mortar and pestle into powder, both with and without using nitrogen freezing of the samples prior to crushing. The powdered material was incubated with 400 mL of 0.5 M EDTA, pH 8.0 for 48 hours. The solution was centrifuged at 6,000 g for 1 hour and supernatant was collected (Sup 1). The pellet was resuspended in 200 mL of 6 M Guanidine-HCl
(GuHCl), pH 7.4 and incubated for 20 hours. The solution was centrifuged at 6,000 g for 1 hour and supernatant was collected (Sup 2).

The next step involved desalting and concentrating of the proteins in the supernatants. Because dialysis can be inefficient and time-consuming, we chose to use a reverse-phase solid-phase extraction column to remove the proteins from the solution and eliminate salts and other unwanted materials. We used 8-carbon chain Solid-phase Extraction columns (C₈ PrepSep Extraction Columns, Fisher Scientific, Fair Lawn, NJ, 07410, Cat. # P452, Lot # 076082), and 18-carbon chain Solid-phase Extraction (Ultra-Clean SPE C₁₈ 200 mg/4 mL, Grace Davison Discovery Science, 2051 Waukegan Road, Deerfield, IL, 60015, Cat. # 505150, Lot # 50295282). The procedure was adapted from Lawrence P. Sandberg’s protocol (personal communication).

Solid-Phase Extraction Column Protocol (C₈ and C₁₈ columns)

The procedure involved preparing the column (Wet, and Equilibrate), putting the sample through the column (Sample), and then washing off salts (Wash), in order to finally elute proteinaceous material (Eluate) for further analysis.

1. Wetting: 3 mL of 100% Acetonitrile (AcN)
2. Equilibrating: 3 mL of 0.1% Trifluoroacetic acid (TFA) in H₂O
3. Sample: Put the whole sample through the Spin columns (100 mL/column, for 400 mL of Sup 1 used 4 columns and for 200 mL of Sup 2 used 2 columns) using vacuum to suck it through or centrifugation.
4. Washing: 12 mL of 5% Methanol (MeOH), 0.1% TFA, in H₂O
5. Eluting: 0.5 mL of 70% AcN, 0.1% TFA in H₂O
All C₈ column supernatant eluates were dried under nitrogen (N₂) flow. For the EDTA supernatant we used 4 columns (100 mL per column), so the total elution volume was 2 mL. For GuHCl supernatant we used 2 columns (100 mL per column), so the total elution volume was 1 mL. We then pooled together both supernatants, for a total pooled volume of 3 mL and dried down the eluate to ~ 0.25 mL in a 50-mL Falcon tube using a flow of N₂. The concentrated eluate was transferred to 2 vials and another 50-100 µL of the elution solution was added to the tube in which they were dried in order to wash it and retrieve the material that was still stuck on the walls of the tube. The C₁₈ column supernatant eluates were also dried under N₂, but they were not combined as the C₈ fractions.

**UV Absorbance**

The UV absorption (Wavescan 200-350 nm) of the sample’s C₈ Eluate solution was measured, using a 10 mm path length quartz cuvette, in the spectrophotometer (NanoPhotometer). Proteins usually show absorbance at 280 nm, due to the presence of the amino acids Phenylalanine (257 nm), Tyrosine (274 nm), and Tryptophan (280 nm) by their characteristic absorbance (Freifelder 1982).

**Total Protein Assay**

We assayed the fossil sample eluates with BCA, which is a quantitative protein assay, and determines the total amount of protein in the solution. We used bovine serum albumin (BSA) as a standard and 96-well micro-plates for the assay. For better accuracy, both the standards and samples were done in triplicate. Because the fossil samples were
in limited amount, we used a miniaturized method for the BCA assay. Instead of using 50 μL of the fossil sample (considering that we only had 300 μL of it), we just used 1 μL (in triplicate) and 4 μL (in triplicate) of each fossil sample, mixed with 51 or 48 μL of diluent (H₂O), and to that we added 50 μL of the BCA Working Reagent in each well. The varied dilution concentrations were all taken into account in the calculations. As for the standards, we used 50 μL of the Standard dilutions and 50 μL of the BCA Working Reagent in each well. For controls (in triplicate) we used the 50 μL aliquots of the 0.5 M EDTA solution, and 50 μL aliquots of the 6 M GuHCl solution, the Wash solution and the Elution solution respectively. As a blank we used Milli-Q Water. After incubating the plate with a plastic cover for over 2 hours at 37°C, we read the UV absorbance at wavelength 562 nm (562 nm is in the visible range).

**Fast Protein Liquid Chromatography**

We used FPLC to sort by sizes the molecules in the elution solution of the fossil samples. We took 150 μL of the concentrated eluate (which was dried down under N₂), then added 80 μL of the elution solution (blank) to fulfill the minimum volume required for injection. The column used was a Superdex Peptide HR 10/30, with a flow rate of 0.5 mL/minute. The equilibration of the column was a ¼ column volume. The sample injection volume was 100 μL. Eluent A was 30% AcN, 0.1% TFA. The fractions were collected in low retention 1.5 mL vials in 0.5 mL aliquots in each.
Matrix-Assisted Laser Desorption/Ionization-Time of Flight

We used MALDI-TOF to confirm the FPLC results regarding the sizes of the molecules in the samples. We took 10 μL of the FPLC eluates and spotted over the spots of the crystallized matrix on the MALDI plate. We also spotted just the matrix, for comparison. Took readings of the major peaks shown in the FPLC results.

Liquid Chromatography Mass Spectrometry

From the FPLC elutions of sample BFW, we collected ~1 µg of protein and placed them in 1.5 mL Eppendorf vials in 15 μL of 0.25% TFA. Samples were analyzed by means of an autosampler, in the Thermo Scientific LCQ Ion-Trap Liquid Chromatography Mass Spectrometer. We used a magic C18 capture column. The resulting “.RAW” files were further analyzed with the software BioWorks, and the resulting putative peptides indicated using the software PEAKS (a DE NOVO sequencing program for interpreting MS/MS results).

Results and Discussion

Protein Extraction

Both EDTA and GuHCl solutions that were extracting the proteins from the fossil samples were brown colored during incubation. After the C8 and C18 column protocols, samples were free from salts, but the eluate remained of a yellowish color.

The C18 eluates of the fossil sample BFW EDTA fraction, after being centrifuged at 6,000 rcf for ~1 minute in a 1.5 mL Eppendorf vial, was seen as being made of two phases. The upper phase was clear and the lower phase was more of a yellowish color.
This separation is seen in mixtures of hydrophilic with hydrophobic molecules, such as mixing water with oil. The presence of oil in this particular extraction can be due to the higher hidrophobicity of the column used – the C_{18} column, as compared to the C_{8} column. The lipid presence in the fossil samples is at least as intriguing as the presence of original proteins in them.

UV Absorbance

Here we provide the UV absorption readings [Fig. 2.01] of both controls and fossil samples. In doing the UV assay of the fossil sample BFW, we saw a small but yet considerable absorbance around 260-280 nm.
Figure 2.01 – UV absorption of protein extractions – UV of (A) Just blank (70% AcN, 0.1% TFA), (B) Fossil BFW, (C) Cow, (D) Chicken, Fossil sample SP08-23 (E) BONE, and (F) its surrounding sediment.
In the comparison of absorptions [Fig. 2.01] the fossil sample SP08-23 shows greater absorption [E] than its immediately surrounding sediment [F]. Figures 2.02-2.10 are the UV readings of controls and fossils. Due to the large number of printouts, we are only including the first result of each sample, with the EDTA and the GuHCl, but not including the many dilutions performed.
Figure 2.02 - UV absorption of Chicken EDTA (left) and GuHCl (right) eluates. Especially in the EDTA fraction there is a prominent peak at around 280 nm, indicative of protein, due to the characteristic absorbance in that region of aromatic amino acids.

Figure 2.03 - UV absorption of Cow EDTA (left) and GuHCl (right) eluates. The same prominent peak at around 280 nm is shown here, which indicates the presence of protein.

Figure 2.04 - UV absorption of SB–Whale EDTA (left) and GuHCl (right) eluates. Peaks seen in the cow and chicken controls around 280 nm are also seen here, but less prominently.
Figure 2.05 - UV absorption of fossil sample BFW EDTA (left) and GuHCl (right) eluates. There is some indication of a bend in the curve of the graph around 280 nm, but the implication of such a small bend may be that there is very little protein in this fossil sample.

Figure 2.06 - UV absorption of fossil sample EFW EDTA (left) and GuHCl (right) eluates. UV absorption apparently does not distinguish the presence of protein in the sample.

Figure 2.07 - UV absorption of fossil sample SP08-20 EDTA (left) and GuHCl (right) eluates.
Figure 2.08 - UV absorption of fossil sample SP08-23-Bone EDTA (left) and GuHCl (right) eluates.

Figure 2.09 - UV absorption of fossil sample SP08-23-Sediment EDTA (left) and GuHCl (right) eluates.

Figure 2.10 - UV absorption of fossil sample SP08-34 EDTA (left) and GuHCl (right) eluates.
The UV absorbance of the fossil samples suggests the possibility that the absorbing amino acids are present in the samples. It shows that there is greater absorbance in the fossil bone than in its adjacent sediment (though this adjacent sediment may have been composed of decaying flesh of the original whale). This seems to indicate that the fossil bone may have protected its original proteinaceous content better than the sediment or soft tissues around it did (such as flesh, baleen, skin, etc.).

When we take into consideration the results of the UV, BCA, and Microscopy, not all samples appear to be very promising as far as protein presence, but a few samples provide encouraging results. We decided to work mostly on sample BFW because it showed promising results in all assays performed.

Total Protein Assay

The total protein results of the Controls (Cow, Chicken and Whale) elutions are shown in figure 2.11.
Figure 2.11 – BCA readings of control samples. Protein yields per gram of sample of the controls, as measured by the Micro BCA assay.
After testing the standards, we proceeded to assay the fossil samples. They showed a much smaller absorbance, relative to the standards. However, if we increased the initial amount of fossil sample, the protein content rapidly reached the higher concentrations seen in the controls.

The total protein (average of the combined supernatants) of the fossil samples is shown in figure 2.12.
Figure 2.12 – BCA readings of fossil samples. Average protein yields per gram of Fossil samples
From these graphs we see that the amount of protein indicated by the BCA assay as being present in the fossil samples vary, but all samples have an overall higher protein amount than the fossil sediment sample (even though the sediment sample was taken from a region right adjacent to the bone sample ~ 0.5 cm away from the edge of the bone).

Therefore the BCA results confirm what the UV absorption results indicated: that small, but yet, detectable amounts of proteinaceous materials are present in the fossil samples.

**Fast Protein Liquid Chromatography**

The fossil sample BFW that was analyzed through FPLC gave indication of the presence of peptides in the sample solution. The peaks were right where they were expected in a control sample peptide graph. The result is shown in Figure 2.13.
Figure 2.13 - FPLC chromatogram of fossil sample BFW. The C₈ elution fractions of EDTA and GuHCl were combined in order that the signal would be stronger. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
Based on this chromatogram and the data obtained from the UV and BCA assays, we may deduce that there are small sized but yet abundant putative peptidic materials in the sample. The majority of it is smaller than 2,000 Daltons, and there appears to be discrete peaks, which may suggest that the peptides vary considerably in size. However there is enough relative abundance of each type or cluster of peptides that individual peaks can be distinguished in the FPLC output.

If the sample had anything larger than 7,000 Daltons, it would come out and be chromatographed around vials A4 and A5. That’s where the large molecular weight material should appear. When we take a close-up look [Fig. 2.14] at that region by enlarging it vertically (notice the scale on the left side), we see that there is a small, but not random, peak at the end of the void volume.
Close-up of FPLC of sample BFW C₈ elution of EDTA + GuHCl combined

Figure 2.14 – FPLC chromatogram of fossil sample BFW showing early peak. The EDTA and GuHCl supernatants were combined in order to improve the protein signal. In the close-up, the smaller peaks can be seen, such as the peak between A4 and A7 labeled “A” pointed out by the arrow. It might reflect the presence of the higher molecular weight materials such as Osteocalcin, some larger collagen fragments, or some unknown protein fragment. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
This peak appears between fractions A5–A6. It may reflect the presence of Osteocalcin in the fossil extract or at least some larger molecular weight protein fragments. The relatively large peaks in the regions from B1–C10, are possibly due to peptides of various sizes. The putative peptidic presence is in agreement with the BCA assay results. We pooled fractions A4-A8 and labeled it “A”, B8-B11 labeled “B”, B12-B14 labeled “C”, and B15-C3 labeled “D”. These fractions are later brought up again in the MALDI-TOF section. The following figure [Fig. 2.15] indicates which fractions correspond to which labels.
Figure 2.15 – Combined fractions of FPLC of sample BFW. Chromatogram of combined EDTA and GuHCl elutions. Letters A-D indicate the fractions that were pooled together in order to proceed to the MALDI-TOF mass spectrometry analysis of this sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
The fractions that were eluted from the FPLC were combined as shown in Figure 2.15, and were afterward dried down in order to concentrate them. These letters (A-D) correspond to samples later spotted in the MALDI-TOF plate and assayed.

Both the previously described BFW extraction and also an earlier extraction (Figure 2.16) show a similar peak in the beginning of the chromatogram, after the void volume. This peak may correspond to larger peptides or almost complete small proteins.
Figure 2.16 – Previous FPLC chromatogram exhibiting early peak. This peak may indicate the presence of higher molecular weight materials in the fossil sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
There is clearly much less material in the region where osteocalcin or some larger protein fragments would be than in the region where smaller peptides would be. This could mean that the majority of the molecules present in the fossil sample extract are very fragmented, but that there is a small presence of the higher molecular weight material. More on this will be discussed in the MALDI-TOF section.

After we had worked with the fossil, we decided to look at the pattern of extant whale protein extract in the FPLC as controls. We realize that the protein profile chromatographed in the FPLC results, and for that matter in any other types of experiments, may show great variance between the fossil and the extant samples because of the numberless factors that may contribute to, or even prevent, protein breakdown.

As for the extant whale control run, the FPLC result is shown in Figure 2.17.
Figure 2.17 - FPLC of an extant whale sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
The modern whale doesn’t show the individual peaks seen in the fossil’s output. This could be because there are too many different protein fragments in this sample, of multiple sizes, which don’t show as individual discrete peaks, but as a large single peak.

Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight

The MALDI-TOF results were very helpful in determining the sizes of the materials present in the sample; more than any other type of experiment we had done before. These peaks are in size agreement with the peaks shown in the FPLC results. The fossil extract resulted in peaks of higher molecular weight than the peaks found only in the matrix blank (the matrix blank was only a matrix spot, without the addition of the sample). The right side of the following figures (Figures 2.18-22) presents the spectrum with peaks from the matrix blank and from the fossil sample BFW. These peaks are plotted by size (x-axis) vs. abundance (y-axis). On the left side, the masses ($m/z$) of the major peaks are listed. Figure 2.18 shows the matrix blank spectrum.
Figure 2.18 - MALDI-TOF of Matrix (Blank) – Only the MALDI matrix used in assisting sample ionization was spotted.
The peaks shown in the matrix blank are related to the sizes of the materials present in the matrix. The largest peaks range up to ~909 Daltons.

Now compare this to the fossil peaks shown in Figures 2.19-2.22. The fossil sample’s spectrum indicates the presence of some extra peaks corresponding to higher molecular weight materials, aside from those present only in the matrix.

The fossil samples shown in Figures 2.19-2.22 are the ones that were subjected to FPLC, which fractions were pooled together (see the FPLC section).
### Figure 2.19 - MALDI-TOF of FPLC Fraction “C” spotting 4 of fossil sample BFW.

<table>
<thead>
<tr>
<th>Mass List</th>
<th>Mass Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>r/mDa</td>
<td>SN</td>
</tr>
<tr>
<td>446.577</td>
<td>9.0</td>
</tr>
<tr>
<td>446.519</td>
<td>9.1</td>
</tr>
<tr>
<td>567.500</td>
<td>8.0</td>
</tr>
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MALDI-TOF of: BFW FPLC Fraction C(4)
Figure 2.20 - MALDI-TOF of FPLC Fraction “C” spotting 5 of fossil sample BFW.
Figure 2.21 - MALDI-TOF of FPLC Fraction “D”, spotting 1 of fossil sample BFW.
### MALDI-TOF of: BFW FPLC Fraction D(3)

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Figure 2.22 - MALDI-TOF of FPLC Fraction “D” spotting 3 of fossil sample BFW.
The peaks shown in the MALDI-TOF output, which were not present in the matrix include: 927, 1033, 1052, 1077, 1096, 1106, 1161, 1287, 1310, 1349, 1498, and 1538 Daltons. These exclude anything below the 908 Daltons, which corresponded to the largest molecular weight peak shown in the matrix blank.

Liquid Chromatography Mass Spectrometry

The overall fragmentation pattern obtained both from the standard used (BSA), and from the fossil sample BFW are shown here. When the BSA fragmentation pattern was compared to the protein database (Mascot, from Matrix Science), there were over 7 protein hits, with dozens of matching sequences. The search was performed by selecting trypsin as the enzyme responsible for the digestion of the BSA protein.

In the analysis of the fossil, we realized we did not have an option of choosing “no enzyme” in the search, although the fossil was fragmented, not by means of a specific enzyme, but by means of unknown processes, of which we have no knowledge. When we performed the search, there were absolutely no significant hits, with zero matching sequences to anything. This makes sense because the search was looking for trypsin digested peptides. This is evidence that there was no cross contamination between the standard and the sample. The implication is that DE NOVO sequencing should only find fossil material and not any contamination from the BSA standard.

Figure 2.23 shows the graphs of the BSA standard fragmentation pattern. Figure 2.24 shows the BSA standard sequence matches by Mascot search against the SwissProt database.
Figure 2.23 - LC/MS of BSA standard. The top part represents the full MS pattern, whereas the bottom section represents the fragmentary MS/MS pattern. The peaks from around 70 minutes up unto 100 minutes are characteristic of peptides.
Figure 2.24 – BSA standard peptide fragmentation match in Mascot. LC/MS matching sequences of BSA in Mascot search when compared to the SwissProt database. BSA standard was digested with trypsin enzyme. There were 7 significant protein hits and dozens of matching sequences.
Figure 2.24 – Continued.
The fossil sample BFW had no significant protein hits when compared against the SwissProt database (Fig. 2.26). In the search, trypsin was specified as the digestive enzyme. If there were trypsin digested peptide hits in the fossil sample, it could be suggested that it was a contamination from the BSA standard. However, because we did not use any enzyme in digesting the fossil material, the absence of trypsin pattern hits supports the idea of non-contamination of the sample with the standard. Nevertheless, even though there were no hits for trypsin digested peptides, BFW displayed secondary (MS/MS) fragmentation (Fig. 2.25) in the place where they should appear in an extant sample. This suggests that BFW contains peptides (not from trypsin) that can be original (from whale), or at least from the sample (whether whale or bacterial, but not from machine contamination). Since we were not able to locate a whale database to compare them to, DE NOVO sequencing seems to be the next required procedure, in order to determine the identity of these peptides.
Figure 2.25 - LC/MS of fossil sample BFW. The upper portion represents the full MS pattern, and the lower portion represents the MS/MS fragmentation pattern. In the same region that peptides appear in non-fossil sample, there is positive signal in the fossil sample. The fragmentation pattern in the lower portion is only due to peptides. If the peaks in fact correspond to peptides, then there are a number of them still present in the sample.
Figure 2.26 – Fossil BFW peptide fragmentation match in Mascot – The fossil sample BFW showed no significant hits, when compared to a database that looked for trypsin digested peptidic material. This supports the non-contamination approach used throughout all the course of this study. Because no trypsin was used to digest the fossil sample, trypsin peptides should not appear, except if there was cross-contamination with standards.
The fragmentation pattern of the fossil is evident in the same region where it usually appears when extant proteins are analyzed. This is very important in determining the nature of the material from fossil BFW analyzed through all the previous experiments and assays. They seem to be suggestive of peptidic material and behave as that. This assay was a further confirmation of the previous steps in the analysis of this fossil sample.

To determine which peptides or sequences they specifically represent will require further research.

Conclusion

The fossil whales of the Pisco Formation are indeed of import, not only from an inorganic, sedimentary perspective, but also from the organic, molecular, and ultrastructural perspective. In addition to the preservation derived from still articulated bones and the presence of fossil baleen in life position, the results of the experiments also suggest a certain level of biomolecular preservation.

There is proteinaceous material in the samples, but the level of it varied. Nevertheless, it is worth of notice that when fossil sediment was compared to fossil bone, even though the sediment sample was located immediately adjacent to the fossil bone, it presented an average protein concentration below that presented by all fossil bones analyzed, including those badly preserved, implying that the fossil bone provided a better environment for biomolecular preservation than its surroundings. Detection of preserved proteinaceous material from the fossil bone samples is supported by the BCA assay, the TLC analysis, the FPLC profile, the MALDI-TOF mass spectra, and the LC/MS
fragmentation pattern (characteristically observed with peptides). In addition to extracted proteinaceous material, we were also able to extract a considerable amount of lipids from the fossil samples. This is consistent with a number of reported lipid findings in fossil samples (Das et al. 1967; Mackenzie et al. 1982; Eglinton et al. 1991; Briggs et al. 2000). The presence of proteinaceous material (and possibly of lipids) suggests that these fossil whales are highly preserved, even down to the biochemical level; this unexpected preservation is now further indicated by reasonable experimentation.

Because of the altered nature of animal remains found in the fossil record, they are far more complex to analyze than extant samples. There is the potential for unexplained changes to happen. Even though we can do repeated experiments, it is not with absolute certainty that conclusions can be drawn. Nevertheless, some conclusion may prove of value.

Unlike the samples that Schweitzer reported from T-rex, with still soft and pliable original tissue (Schweitzer et al. 2005), our samples, after partial deminERALIZATION remained brittle, implying that much of the organic material had been replaced by mineral components. In spite of that, we still observed evidence of proteinaceous, as well as lipid material.
References


Embery, G., A. Milner, R. J. Waddington, R. C. Hall, M. S. Langley and A. M. Milan (2000). "The Isolation and Detection of Non-Collagenous Proteins from the


CHAPTER THREE
MATERIALS AND METHODS

In this section we describe the methods used in this study in search for ancient biomolecules. We went from general to very specific assays: light microscopy, and then, Scanning Electron Microscopy for more detail. After that, we used biochemical methods to describe the search for biomolecules. The methods we used are listed below.

1. **Light Microscopy** of samples in order to have a better understanding of the degree of the visual preservation of the samples.

2. **Scanning Electron Microscopy** of partially demineralized fossil samples, in order to verify the level of ultrastructural preservation.

3. **Ultra-violet analysis** of the absorbance values consistent with aromatic amino acids.

4. **Micro-Bicinchoninic acid Assay** was employed in order to determine the protein concentration of the sample extracts.

5. **Fast Protein Liquid Chromatography**, enabled improved resolution of protein and peptide fragments from sample extracts.


7. **Dansylation** of amino acids and **Thin-Layer Chromatography**, to see the amino acid distribution of the samples, and confirm the results from BCA, FPLC and MALDI-TOF, supporting peptide and amino acid presence in sample extracts.
**8. Liquid Chromatography Mass Spectrometry**, which is potentially able to give putative peptide sequences based on MS/MS of Collision-induced dissociation (CID).

**General Sample Preparation**

Each sample was initially demineralized with 0.5 M EDTA (ethylenediaminetetraacetic acid, pH 8.0, 0.22 μm filtered). The EDTA acts as a chelating agent, which “sequesters” metal ions such as Ca²⁺ and Fe²⁺, breaking the structure of minerals, and allowing proteins to be released into the aqueous solution. Although many proteins are water soluble, there are some, like the scleroprotein *collagen*, that are generally hydrophobic, and tend to aggregate together. These proteins need to be denatured in order to go into solution. In order for that to happen, we used a 6 M solution of the strong denaturing agent Guanidine-HCl (GuHCl, pH 7.4, 0.22 μm filtered). The procedure we used was adapted from Schweitzer (2007a).

For microscopy experiments, only EDTA-dependent demineralization was performed. For all other experiments the demineralization step was followed by the protein denaturation step with GuHCl.

For positive controls we had: cow and chicken (both obtained from a local market), extant whale (obtained from Raul Esperante) and a 20-year after death weathered whale jawbone (a Santa Barbara Museum of Natural History, CA, specimen provided to us by Paul Collins [pcollins@sbnature2.org], who serves as the curator for the Museum).

Fossil samples included whale bones and sediment samples from the Pisco Formation, Peru. Most of the analyses involved the sample labeled “BFW”. This sample
was part of a rib from a fossilized baleen whale, cf. Balaenopteridae, found at GPS location 18 L 8411328/422633 (datum South American ’69), which is in the upper, diatom-rich, part of the Miocene/Pliocene Pisco Formation in Peru. The sample was originally collected by Leonard Brand, from Loma Linda University, CA, and bore the field label: “CBa-1 Whale #20, W20-99-015”.

**Light Microscopy**

About 1 g of the sample “BFW” was crushed into small pieces (1 mm² or less) with a steel mortar and pestle and subjected to demineralization using 10-20 ml of 0.5 M EDTA, pH 8.0, for 3-5 days in a Petri dish, without shaking (to prevent damage to fragile structures in the samples by movement). After partial demineralization, visible changes were observed and water was substituted for the EDTA in order to stop further demineralization. Samples were surveyed using a stereoscope (Bausch and Lomb Stereo Zoom inspection scope, Rochester, NY) with a magnification of 0.7–3.0 X.

**Scanning Electron Microscopy**

Following initial work with light microscopy, further analysis was pursued with the SEM for added detail. We began another demineralization procedure, which lasted for about 5 days. When demineralization seemed to effect no more visible changes in the sample, we stopped it by introducing water and removing the EDTA solution from the Petri dish. The partially demineralized bone fragments were collected from the Petri dish and transferred to the SEM’s specimen holder using an adapted 200 µl pipette tip which had the foremost part of the tip severed, so that the opening would be large enough,
reducing physical damage to the fragile demineralized structures, as they were sucked up the tip while being transferred. All specimens were Au/Pd coated for 60 seconds and then placed in the SEM (Tescam) for analysis in vacuum.

**X-ray Diffraction**

About 2 g of sample BFW was demineralized with 0.5 \( M \) EDTA, pH 8.0, for 2-5 days. When no further noticeable demineralization was happening, we collected the vessel-like structures that remained on the Petri dish and subjected them to XRD.

**Biomolecules Extraction**

We performed multiple extractions of proteins from the fossils. At first, we started with a relatively small amount of fossil bone (~2 g), but kept increasing it, as we better understood the protein concentration present in the samples. We surveyed an array of fossil bones in order to determine the most adequate sample for conducting further experiments. We experimented with dialyzing in order to retrieve the proteins from the demineralization solution, but opted for another method. One that seemed to be more straightforward, and less labor demanding – C\(_8\) spin columns.

**Protein Extraction of 2-gram Samples**

The initial amount used for a general survey was about 2 g of each of the 10 fossil samples. All samples were frozen with liquid nitrogen, and ground to powder using a steel mortar and pestle. The powdered material was demineralized with 10 ml of 0.5 \( M \) EDTA overnight, and centrifuged (3,340 G). The supernatant was collected (Sup 1). Then
another aliquot of 10 ml of 0.5 M EDTA was added to the pellet and incubated with shaking for 3 days. The sample was centrifuged again and the supernatant was collected (Sup 2). Then 5 ml of 6 M GuHCl was added to the pellet, and incubated at 60 °C overnight. The sample was centrifuged and the supernatant collected (Sup 3). We collected the proteins using the C₈ SPE columns (Fisher), and eluted them in 70% AcN, 0.1% TFA. We proceeded to assay the eluates with UV absorption and to measure protein signal by means of the BCA assay, as will be described below. Separately we performed tests on extant control bones of cow, chicken and whale.

Protein Extraction of 20-gram Samples

Another extraction was done using a larger amount of the bone samples (~20 g). Samples were frozen with liquid nitrogen, and ground to powder using a steel mortar and pestle. As in the previous extraction, the powdered material was demineralized with 100 ml of 0.5 M EDTA overnight, centrifuged (3,340 G), and the supernatant collected (Sup 1). Then another aliquot of 100 ml of 0.5 M EDTA was added to the pellet and incubated with shaking for 3 days. The sample was centrifuged again and the supernatant was collected (Sup 2). Then 50 ml of 6 M GuHCl was added to the pellet, and incubated at 60 °C overnight. The sample was centrifuged and the supernatant collected (Sup 3). Instead of using C₈ Spin columns, we dialyzed the supernatants against nano-pure water using a 2000 M.W. cutoff membrane for 3 days, changing the water 3–5 times at 4°C (in a 4 L container). The contents were transferred to several tubes and frozen (-20°C or -80°C). The tube lids were removed and the tubes were closed with parafilm. Holes were poked in the parafilm in order that the water may be sublimated through them, and the
contents were lyophilized (trap temperature -50 °C, pressure 7–10 P) for several days until dryness. The lyophilized material was taken and assayed with the same steps that were followed after the C8 chromatography.

Protein Extraction of a 55-gram Sample

Finally, once we knew, from the previous results, which sample probably had the highest protein preservation (sample BFW), we used up what we could of this sample in a final extraction (~55 g). This time we did not freeze the samples prior to powdering, and the results were still the same. The sample was ground to powder using a steel mortar and pestle. But in contrast to the previous extraction, we only used 1 EDTA extraction (2 days), and one GuHCl extraction (20 hours). To the 55 g of powdered material we added 400 ml of 0.5 M EDTA, and incubated at room temperature for 2 days with shaking. Then we centrifuged it at 6000 G for 1 hour, and collected the supernatant. Then we incubated it with 200 ml of 6 M GuHCl for 20 hours at 60 °C with shaking. We centrifuged it again at 6000 G for 1 hour and collected the supernatants, which were then put through C8 extraction columns. These supernatants were then analyzed with more extensive experiments than all prior samples, except the controls. These steps are summarized in Figure 3.01.
Figure 3.01 – Summary diagram illustrating C8 column procedure for protein extraction. (1) Both salts (green) and proteins (yellow) are together in the solution, but we want to get rid of salts and keep the proteins. The solution is put through the C8 column. (2) Salts are flowed through, and the proteins are retained. (3) Proteins are finally eluted from the column and saved.
Protein Capture by C₈ Solid-Phase Extraction Columns

Proteins from the supernatants were first captured using the PrepSep C₈ Solid-Phase Extraction Columns (C₈ PrepSep Extraction Columns, Fisher Scientific, Fair Lawn, NJ, 07410, Cat. # P452, Lot # 076082), also referred to as “spin columns”, according to the following protocol:

- To the column, we added 3 ml of wet solution: 100 % AcN and centrifuged it for 1 minute. Discarded the flowthrough.
- Added 3 ml of equilibrate solution: 0.1 % TFA and centrifuged it for 1 minute. Discarded the flowthrough.
- Flowed the whole supernatant through the column. Saved the flowthrough.
- Added 12 ml of wash solution: 0.1 % TFA, 5 % MeOH and centrifuged for 2 minutes. Discarded the flowthrough.
- Added 0.5 ml of elute solution: 70 % AcN, 0.1 % TFA. Centrifuged for 1 minute. Saved the eluate.

Protein Capture by C₁₈ Solid-Phase Extraction Columns

Following the C₈ extraction, we decided to capture everything we could of proteins/peptides from the supernatants that flowed through (the “flowthroughs”) of the C₈ spin columns. So we decided to use the C₁₈ extraction columns (Ultra-Clean SPE C₁₈ 200 mg/4 ml, Grace Davison Discovery Science, Deerfield, IL, 60015, Cat. # 505150, Lot # 50295282), for their higher affinity to non-polar molecules:

- To the column, we added 3 ml of wet solution: 100 % AcN and centrifuged it for 1 minute. Discarded the flowthrough.
• Added 3 ml of equilibrate solution: 0.1 % TFA and centrifuged it for 1 minute. Discarded the flowthrough.
• Flowed the whole supernatant through the column. Saved the flowthrough.
• Added 12 ml of wash solution: 0.1 % TFA, 5 % MeOH and centrifuged for 2 minutes. Discarded the flowthrough.
• Added 0.5 ml of elution solution: 70 % AcN, 0.1 % TFA. Centrifuged for 1 minute. Saved the eluate.

Therefore, we collected everything we thought possible to collect. In one of the column elutions (the C_{18} elution from the BFW EDTA fraction), after we centrifuged the eluate, we were surprised to see that the liquid inside the vial was composed of two phases! There was a clear part (the upper), which was divided by a distinct line from the more yellow colored line (the lower). This could indicate that the sample still contains lipids, which were captured by the C_{18} column. The reason we had never seen this before is that we had never used the C_{18} columns before to capture materials from the BFW demineralizing fractions. For most of the experimentation time we had only used C_{8} columns, which are less hydrophobic than the C_{18}, and therefore, less able to capture lipids than the C_{18} columns.

**UV Absorbance**

The UV absorption (Wavescan 200-350 nm) of the sample’s C_{8} **Eluate** solution was measured, using a 10 mm path length quartz cuvette, in the spectrophotometer (NanoPhotometer). Proteins usually show absorbance at 280 nm, due to the presence of the amino acids Phenylalanine (257 nm), Tyrosine (274 nm), and Tryptophan (280 nm).
by their characteristic absorbance (Freifelder 1982). We took repeated measurements of each sample, with several dilutions of each, but we report the most prominent results of each sample.

**Bicinchoninic Acid Assay**

Because UV absorbance can also be due to DNA, or other unknown factors, and not just to protein presence, we performed more specific tests in order to better determine the nature of the material preserved.

We assayed the fossil sample eluates with BCA to determine the total level of protein in the solution. We used BSA as the standard, and the 96-well Polystyrene assay microplate (Corning, Lowell, MA) for assay preparation, incubation, and reading. We assayed both samples and standards in triplicate. We used 75 µL of the BCA Working Reagent (prepared using 25 parts of solution A, 24 parts of B and 1 part of C) and 75 µL of sample or standard or blank on each well. After incubating the plate with a plastic covering for at least 2 hours at 37 °C, we read the UV absorbance at wavelength 562 nm in a µQuant machine (Bio-Tek Instruments, Inc. Winooski, VT).

**Gels**

SDS-PAGE gel assays of both the controls and fossils were performed. In the protocol used, aliquots of the samples were dissolved in HEPES buffer (20 mM HEPES, 150 mM NaCl), and transferred to 1.5 ml vials. To the vials was added NuPAGE LDS Sample (Bromophenyl blue). Then the vials were heated for 5 minutes at 88°C, their
content was transferred to the gel wells and run. The gels used were 4-12% SDS-PAGE (Gradient NuPAGE 4-12% Bis-Tris-Gel).

Samples were subjected to 2 different staining methods: Coomassie Blue and Silver stains. The Coomassie Blue comes in a solution already prepared. We added that solution to a container and placed the gel in it. We put the container in a shaker, and left it staining for over 2 hours.

The silver stain method requires considerably more effort, and it is not as straightforward to apply as the Coomassie Blue stain, but it is more sensitive, and therefore, can detect lower amounts of protein. The protocol for silver staining is described below:

Silver Stain Protocol (Mass Spectrometry Compatible)

1. Fix gel in 100 mL of 20% ethanol, 1% acetic acid for 1 hr.
2. Wash gel in 20% ethanol for 10 min.
3. Pre-treat gel with 100 mL 0.02% sodium thiosulfate for 1 min.
4. Decant and rinse gel in Milli-Q water for 1 min.
5. Add 100 mL of 0.1% silver nitrate solution and agitate for 15-20 min.
6. Decant and rinse gel in Milli-Q water for 1 min.
7. Decant and add 100 mL of developing solution (2% sodium carbonate, 40 mL 37% formaldehyde). Develop until protein spots are visible (5-10 min).
8. Stop developing with 5% acetic acid (5-10 min) and replace with Milli-Q water.
After removing the gel from the solution, we inspected the gel surveying it for any visible bands.

**Fast Protein Liquid Chromatography**

The initial preparation of the fossil sample “BFW” for FPLC and MALDI-TOF is described in the “Protein Extractions” section. The specific amount used from the fossil sample was 55 g. EDTA and GuHCl extraction supernatants were put through the C₈ Spin column and eluted in 0.5 ml of the elution solution. There were 4 columns for the EDTA and 2 columns for the GuHCl, so that the combined elution volume was 2 ml from the EDTA extraction and 1 ml from the GuHCl extraction (a total of 3 ml).

These 3 ml were combined and divided into two 50-ml falcon tubes, in order to concentrate them under a laminar flow of nitrogen. They were concentrated from 1.5 ml down to about 200 µl on each tube, and then transferred to 1.5 ml Eppendorf vials. After the transfer, we added 100 µl of the elution solution (used in this case as a re-suspension solution) to each of the 50-ml tubes in order to retrieve, as much as possible, the molecules that might have been stuck to the walls (concentrating the sample decreases its solubility). After shaking them well, we transferred the 100 µl suspension solutions to the contents already in the Eppendorf vials. The total volume in each vial reached about 300 µl of concentrated sample (fossil stock).

We took 1 of these vials and dried it down still further to 150 µl. Then we assayed the sample with FPLC using a Superdex Peptide HR 10/30 column, at a flow rate of 0.5 ml/minute, with buffer of 30% AcN, 0.1% TFA. We used 2 column volumes for replacing the column’s storage medium and ¼ column volume before each sample for
equilibration of the column (with 5 ml of void volume and another 5 ml of equilibration). The best resolution of this type of column is for molecules whose weight ranges from 7,000-300 Daltons.

This type of column is especially useful for peptidic analysis, and anything above 7000 Daltons will likely flow through first as a peak early in the elution, along with the injection front, right after the excluded volume (around 6.25 ml).

In preparing the sample to be put through the column, we realized we needed over 200 µl in order to have a full 100 µl injected in the column, but we had dried it down to 150 µl already. So we added 80 µl of the running buffer to the sample vial, shook well, and centrifuged that vial at 11,000 G for 10 minutes in order to get rid of any particular matter. We then injected 100 µl of the sample in the column.

The FPLC fractions were eluted in 0.5 mL aliquots into 1.5 mL Eppendorf vials and were afterward dried down in the speed vac. The concentrated solutions were spotted onto the MALDI-TOF plate for mass spectrometry.

The settings used for the FPLC column procedure are shown in Figure 3.02.
Figure 3.02 – FPLC settings printout – Settings used in the protocol used in the FPLC analysis of the fossil sample BFW.
Besides the fossil, we used a protein extraction from an extant whale, extracted in the same manner as the fossil. In order to use the same peptide column, which was used for the fossil, we decided to use a filter membrane that would remove anything larger than 30,000 Daltons, so that no excessively large peaks would appear in the beginning of the chromatogram. We just wanted to see what peptides were present in the extant whale, without the necessity of digesting its proteins. We put the filtrate through the FPLC column and chromatographed it using the same settings described earlier in the fossil procedure. Then we took aliquots from a few of these fractions and spotted them on MALDI-TOF to see their profile.

**Matrix-Assisted Laser Desorption/Ionization-Time of Flight**

In order to determine the sizes of the molecules in the protein extract, we proceeded to assay it with MALDI-TOF. We took the C₈ spin column EDTA 1, EDTA 2, and GuHCl eluates, some which were and some which were not put through FPLC, and spotted them in the MALDI plate. Again we will not show all the data collected, because there were many readings (which can be found in the lab books), but we will show the most relevant ones. The protocol used was the following:

**MALDI-TOF Protocol**

1. Wash plate and dry
2. Deposit 0.5 µl of 50 mM ammonium bicarbonate
3. Place on hot plate for 10 seconds
4. Deposit 0.5 µl of matrix solution (HCCA [α-Cyano-4-hydroxycinnamic acid], SA [sinapinic acid] or DHB [2,5-dihydroxybenzoic acid])

5. Place on hot plate for 10 seconds

6. Deposit 0.5 µl of sample solution

7. Place on hot plate for 10 seconds and analyze

A variation of this protocol was also used, skipping steps 2 and 3. Both linear and reflected TOF were used. Besides the fossil, we also assayed cow, chicken and whale as controls.

**Dansylation and Thin-Layer Chromatography**

In order to have a better appreciation of the nature of the material indicated in the FPLC and MALDI-TOF outputs, we assayed the samples with Polyamide TLC. This aids in confirming the proteinaceous properties of the sample extracts, resolved by FPLC or MALDI-TOF into distinct peaks. In this assay, the amino acids are dansylated and migrate through the thin-layer plate carried by the solvent phase (hydrophilic or hydrophobic). The solvents are “run” in 2 dimensions, with the first dimension using a hydrophilic solvent, and the second dimension employing a hydrophobic one. Because the dansyl label is highly fluorescent under UV light, even a low amount of amino acids (a few nmols) is enough for detection through this method. Furthermore, the amino acids travel at certain distances in the plate. The relative position of the spots in the TLC plate can be correlated with the traveled distances of amino acid standards.

The method we used was adapted from Low et al. (1980) and Van Buskirk et al. (1980). We made a few changes to the methods used by these researchers because some
of the new resources available today for assays may not have been available 30 years ago. One of the changes was from Porapak to C\textsubscript{18} Extraction columns. We experimented with both columns, and the C\textsubscript{18} column gave a much better result than the Porapak (see comparison of C\textsubscript{18} vs. Porapak below).

As standards we used glutamic acid (Sigma, Cat. # G1251, Batch # 079K01021), \(\gamma\)-carboxyglutamic acid (also known as “Gla”, from Sigma, Cat. # C4147, Batch # 089K1177), and Osteocalcin (bovine, MP-Biomedical, Solon, OH 44139, Cat. # 194940 Lot # 9378j), as well as the blanks (DNS-Cl blank and Elution solution blank). The DNS-Cl was from Invitrogen, Cat. # D21, Lot # 513354. Below is the step-by-step outline used in the adapted method.

For the Glu and Gla standards, we skipped the hydrolysis steps because they were individual amino acids. We prepared a 10 mM Glu or a 20 mM Gla solution in 100 mM Na\(\text{HCO}_3\) respectively, and stored each at -20°C. From the stock solution we took the equivalent volume containing \(~1\) nmol of each amino acid standard for labeling with a dansyl fluorophore.

Since acid hydrolysis also hydrolyses the extra carboxyl group in the \(\gamma\) position of the Gla amino acid R group, we had to use alkaline hydrolysis for the osteocalcin and for the fossil material. The hydrolysis vial was a 5 ml Reacti-vial Small Reaction Vials (Thermo Scientific, Rockford, IL, 61101, Prod. # TS–13223, Lot # LA12718830), with a Miniert valve (Thermo Scientific, Prod. # TS–10135). We pipetted into it, 100 \(\mu\)l of 2 M NaOH and 1 \(\mu\)l of a 10 \(\mu\)g/\(\mu\)l Osteocalcin solution (\(~1.7\) nmol). We then removed O\(_2\) from the Reacti-vial by introducing nitrogen gas into it, and letting it run for 3 minutes.
The vials were then incubated at 108°C for 22 h. After hydrolysis, we neutralized it to pH 7.5 and made it to 100 mM NaHCO₃.

Standards or fossil solutions were mixed with equal volumes of 750 µg/ml DNS-Cl in acetone, and incubated 4 hours in the dark. Then the solutions were dried down in the speed vac to remove acetone. Then these were dissolved with 500-1000 µl 0.1% TFA in water. These preparations were then applied to the C₁₈ extraction columns (Ultra-Clean SPE C₁₈ 200 mg/4 ml, Grace Davison Discovery Science, Deerfield, IL, 60015, Cat. # 505150, Lot # 50295282). Free amino acids were adsorbed to these C₁₈ columns, The columns were employed using the following amino acid retrieval protocol: wetted the column with 3 ml of 100% EtOH, equilibrated the column with 3 ml of 100 mM HCl, then put the sample through the column (saved apart the flowthrough), washed the column with 12 ml of 100 mM HAc in order to remove the salts, eluted the sample from the column with 0.5 ml of a solution of acetone, EtOH, water (4:5:1).

The eluates were evaporated to dryness and redissolved in 10 µl of EtOH. The samples were spotted on the Polyamide TLC plates. Used 20 cm square polyamide sheets (Polyamide-6 sheets TLC plates, polyester backed, thickness 100 µm, Sorbent Technologies, Atlanta, GA 30366, Cat. # 3522026, Lot # 010113K). Figure 3.03 shows the Polyamide sheet solvent and spotting layout.
Figure 3.03 - Polyamide sheet solvent and spotting layout.
Added 0.5 μl aliquots of sample at the spot origin (2 cm from each edge of the polyamide sheet), let it dry, repeated spotting until all 10 μl were applied to the sheet, and spotted several combinations in different plates: DNS-CI blank, elution solution blank, just Gla, just Glu, Gla+Glu, Osteocalcin, and Fossil.

In the first running dimension we used the polar solvent: 100 ml of 1.35% formic acid, and placed the plates in the TLC chamber, which had the solvent and closed the chamber with its lid. Then we let the solvent run up the plate for 4-5 hours, removed the plates from the chamber, let them dry and imaged them. We then removed from the chamber the first dimension’s solvent and added to it the second dimension’s solvent: 100 ml of n-heptane, n-butanol, glacial acetic acid (3:3:1). We placed the plates in the chamber and let the solvent run up the plates for 2.5 hours, removed the plates from chamber, let them dry and imaged them.

C_{18} vs. Porapak Columns

Which method is better for separation the DNS-amino acids (any amino acid) from the DNS-hydroxide (which is not linked with any amino acids)? We tested 4 methods or combinations using the Glutamate (Glu) standards. After Glu had been dansylated, we took the equivalent volume of 100 nmols of Glu for testing each method. We used 2 C_{18} columns and 2 Porapak columns. For the first C_{18} column we used the protein retrieval protocol: wetted with 3 ml of 100% AcN, equilibrated with 3 ml of 0.1% TFA, then the sample was put through. It was further washed with 12 ml of 5% MeOH, and eluted in 0.5 ml of 70% AcN. On the second C_{18}, we used the standard amino
acide retrieval protocol described in the previous pages. For the Porapak columns we also used the standard amino acid retrieval protocol.

The elution of each of the columns was dried and taken up in 10 µl of EtOH. We put 9 spots in each sheet (duplicated the same setup for each sheet): 1 spot in the center of the lower part of the plate from a DNS-Cl blank, that had not been put through the column served as a control, then one from the C₁₈ eluate, which had been processed with the protein protocol, and 1 from a C₁₈ eluate that had been processed with the amino acid protocol, then 2 spots from each of the Porapak columns. All spots were done using just 0.5 µl of each eluate, and they were repeated in 2 plates (one plate was run only with the 1st dimension’s solvent, the other was only run with the 2nd dimension’s solvent).

We chose to use the C₁₈ column with the amino acid retrieval protocol (see results section). We spotted Gla standards, Gla+Glu standards, and Osteocalcin standards on the TLC plates. On the Gla plate we spotted about 2.5 nmols of a DNS-Gla stock solution; on the Glu plate we spotted about 5 nmols of the DNS Glu stock solution; on the Gla+Glu plate we spotted 2.5 nmols of DNS-Gla and 5 nmols of DNS-Glu stock solutions on the same spot. On the osteocalcin plate we spotted 1.7 nmols of osteocalcin (previously hydrolyzed and dansylated).

For the fossil samples we took some 80 µl of the fossil stock (described in the FPLC results section), and hydrolyzed with 100 µl of 2 N NaOH for 22 hours. Then we neutralized the hydrolysate to pH 7.4, and dansylated it for 4 hours with equal volumes of 750 µg/ml DNS-Cl in acetone. Then we put it through the C₁₈ column with the protein retrieval protocol and eluted in 0.5 ml of 70% AcN, 0.1% TFA. Then we dried down the flowthrough of the column, put it again through the C₁₈ column, and re-eluted with the
protein retrieval protocol. Took 0.5 ml of the DNS-Cl solution blank and put it through the C_{18} column using the protein retrieval protocol as well. We dried down both samples, spotted in the polyamide TLC plate, ran the plate in the solvents of the two dimensions, and imaged the results.

Liquid Chromatography Mass Spectrometry

From the FPLC elutions of sample BFW, we collected ~1 \mu g of protein and placed them in 1.5 mL Eppendorf vials in 15 \mu L of 0.25% TFA. Samples were analyzed by means of an autosampler, in the Thermo Scientific LCQ Ion-Trap. The resulting “RAW” files were further analyzed with the software BioWorks, and the resulting putative peptides indicated using the software PEAKS (a DE NOVO sequencing program for interpreting MS/MS results).
CHAPTER FOUR

RESULTS

The results follow the same sequence in which they were introduced in the section “Materials and Methods”. Because of the abundance of tests and repeated experiments, we report here the most prominent results. If there were several repeats of the same step for one sample, we only show one of the results for the sake of limited space.

Light Microscopy

As the EDTA began working, the fossil bone slowly disappeared. After the bone matrix was mostly consumed away, what was left was whatever the EDTA could not dissolve. Figure 4.01 shows the reddish structures in the white bone matrix. Figures 4.02-4.03 show the liberated vessel-like structures.
Figure 4.01 - Fossil bone in the progress of demineralization. Vessels (reddish tubular structures) protrude from the bone matrix, and are not consumed by EDTA, while the matrix continues to disappear.

Figure 4.02 – Fossil whale vessel casts liberated from bone fragments that had been demineralized with EDTA (under reflected light).

Figure 4.03 – Branching fossil whale vessel liberated from demineralized bone (using transmitted light). The images seem to suggest that the vessels are hollow (red arrow) and made of some fibrous material (yellow arrow).
These results were very surprising. The dissection microscope revealed structures that very much resemble branching blood-vessels, red-colored, many of them lying on the bottom of the Petri dish [Fig. 4.02-4.03], but several of them still extending through the demineralizing fossil bone fragments [Fig. 4.01]. As the EDTA consumed the bone, the vessel-like structures remained in position, and more prominently protruded from the fossil bone matrix.

The vessel-like structures were straight or branching and of various thicknesses. They behaved very much like glass. They would crack as we tried to pick them up with any metal device, but would not be consumed by the EDTA, even after many days.

We then examined thin-sections of other fossil whale bones from the same Formation. The microscopy of the thin-sections revealed what clearly resembled Osteocyte lacunae [Fig. 4.04], and also the porous structure seen in spongy bone.

**Scanning Electron Microscopy**

Fossil sample BFW showed surprising structural preservation. It displayed vessel and cell-like structures preservation [Fig. 4.05-4.14]. However, osteocyte and vessel shapes were also seen in other fossil samples [Fig. 4.15-4.20].
Figure 4.04 - Fossil whale bone thin sections showing Osteocyte lacunae pattern (under reflected light).
Figure 4.05 - Branching vessel structures after demineralization of fossil BFW bone fragment with EDTA for 3 days.

Figure 4.06 - Interconnected vessel structure remains from demineralized fossil BFW.
Figure 4.07 – BFW fossil blood vessel.

Figure 4.08 – Incomplete demineralization of BFW fossil bone. Vessels protruding outward from bone aligned in the same direction (see arrow).
Figure 4.09 - BFW close-up of vessels protruding out of demineralizing fossil bone.

Figure 4.10 – BFW close-up of fossil vessel displaying an osteocyte cell at the inferior part of the vessel (arrow).
Figure 4.11 – BFW fossil bone blood vessel surface covered with osteocytes (arrows).

Figure 4.12 – Fossil osteocyte close-up.
Figure 4.13 - Osteocytes partially embedded in demineralizing fossil bone matrix.

Figure 4.14 - Close-up of the fossil osteocyte and its canaliculi (indicated by arrows) extending downward through the matrix.
Figure 4.15 - Partially exposed fossil osteocyte from sample EFW.

Figure 4.16 – Fossil osteocyte (see arrow) associated with vessel structure from sample SP08-20.
Figure 4.17 – Fossil osteocytes around vessel structure from sample SP08-20.

Figure 4.18 - Completely exposed fossil osteocyte from sample SP08-34.
Figure 4.19 - Multiple (1) osteocytes from sample SP08-34.

Figure 4.20 – Multiple (2) osteocytes from sample SP08-34.
X-Ray Diffraction

The XRD results of fossil sample BFW [Fig. 4.21] indicated with a great degree of confidence that the majority of the material remaining after demineralization, of which the blood vessels were made of, is quartz. Below is the graph showing the quartz pattern for this sample.

Biomolecules Extraction

Besides the results showing a certain amount of proteinaceous material per gram of demineralized fossil material (as shown by BCA), there was also an indication of lipids in the C\textsubscript{18} extraction of sample BFW (EDTA supernatant). Because we wanted to collect as much proteinaceous material from the sample as reasonably possible, we put the demineralized solutions (EDTA and GuHCl supernatants) through the C\textsubscript{8} columns, which captured the proteins and peptides in the solution. After the sample flowed through the C\textsubscript{8} column, we put the flowthrough again through a still more hydrophobic column, the C\textsubscript{18} columns. In the “C\textsubscript{18} elution from the BFW EDTA fraction”, after we had partially dried it under nitrogen flow to about 200 µL and after we centrifuged this dried down solution at 10,000 rcf for 2 minutes, we were surprised to see that the liquid inside the vial was composed of two phases! The upper phase was clearer and the lower phase had more of a yellowish appearance. The separation was clearly visible, and the two phases were distinctly separated.
Figure 4.21 - XRD results of the remnants of the demineralized fossil bone. Included were blood vessels and some unidentified material that was not dissolved by EDTA.
This may be an indication that the sample still contains lipids, which were captured by the C\textsubscript{18} column (more hydrophobic than the C\textsubscript{8} column). For most of the time in this research we had only used C\textsubscript{8} columns, which are less hydrophobic than the C\textsubscript{18}, and therefore, less able to capture lipids than the C\textsubscript{18} columns. Only at the final weeks we used the C\textsubscript{18} and realized that lipids might also be present in the fossil samples.

**UV Absorbance**

Figures 4.22-4.23 show the UV absorption readings of both controls and fossil samples. In doing the UV assay of the fossil sample BFW, we saw a small absorbance around 260-280 nm.

Figures 4.24-4.32 show the UV readings of controls and fossils. Due to the large number of printouts, we are only including the first result of each sample, with the EDTA and the GuHCl, but not including the many dilutions, which were performed.
Figure 4.22 – UV absorption of fossil sample BFW (EDTA and GuHCl supernatants).

Figure 4.23 – UV absorption comparison of fossil bone SP08-23 and its surrounding sediment.
Figure 4.24 - UV absorption of Chicken EDTA (left) and GuHCl (right) eluates. Especially in the EDTA fraction there is a prominent peak at around 280 nm, indicative of protein, due to the characteristic absorbance in that region of aromatic amino acids.

Figure 4.25 - UV absorption of Cow EDTA (left) and GuHCl (right) eluates. The same prominent peak at around 280 nm is shown here, which indicates the presence of protein.

Figure 4.26 - UV absorption of SB–Whale EDTA (left) and GuHCl (right) eluates. Peaks seen in the cow and chicken controls around 280 nm are also seen here, but less prominently.
Figure 4.27 - UV absorption of fossil sample BFW EDTA (left) and GuHCl (right) eluates. There is some indication of a bend in the curve of the graph around 280 nm, but the implication of such a small bend may be that there is very little protein in this fossil sample.

Figure 4.28 - UV absorption of fossil sample EFW EDTA (left) and GuHCl (right) eluates. UV absorption apparently does not distinguish the presence of protein in the sample.

Figure 4.29 - UV absorption of fossil sample SP08-20 EDTA (left) and GuHCl (right) eluates.
Figure 4.30 - UV absorption of fossil sample SP08-23-Bone EDTA (left) and GuHCl (right) eluates.

Figure 4.31 - UV absorption of fossil sample SP08-23-Sediment EDTA (left) and GuHCl (right) eluates.

Figure 4.32 - UV absorption of fossil sample SP08-34 EDTA (left) and GuHCl (right) eluates.
Bicinchoninic Acid Assay

The BCA method, which measures the total protein of a given solution was very helpful, and extremely consistent. The results for both controls and fossil samples are shown. The average protein yield in Cow, Chicken and Whale controls are shown in Figure 4.33. The detailed individual amounts of protein in each supernatant of the control samples are shown in Figure 4.34.

After testing the standards, we proceeded to assay the fossil samples, but with experiments several weeks apart. The fossil samples showed a much smaller absorbance [Fig. 4.35-4.36], relative to the standards, but increased as the amount of starting fossil material increased.

The detailed amounts of protein in each supernatant of the fossil samples are shown in Figures 4.37-4.39.
Figure 4.33 - Protein yields per gram of sample of the Controls, as measured by the Micro BCA assay.
Figure 4.34 - BCA assay of protein amounts in the Eluates of Cow, Chicken and Whale. For samples “Whale S1 and Whale S3” the absorbance was so high that the instrument was not able to give a number. The purple color was darker than the 200 µg/ml BSA standard, so it follows that the Whale S1 and S3 had higher protein amounts than 200 µg/ml.
Figure 4.35 – Average protein yields per gram of Fossil samples
Figure 4.36 - Protein yields per ml of elution solution of the Fossils
Detailed BCA reading results from fossils EDTA 1 Supernatant:

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Figure 4.37 - BCA assay from fossil samples (Supernatant EDTA 1).
Detailed BCA reading results from fossils EDTA 2 Supernatant:

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Figure 4.38 - BCA assay from fossil samples (Supernatant EDTA 2).
Detailed BCA reading results from fossils GuHCl Supernatant:

Figure 4.39 - BCA assay from fossil samples (Supernatant GuHCl).

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Gels

Gel results from the controls and from the fossil sample BFW are shown in Figure 4.40-4.45. The first gel [Fig. 4.40] is from cow and chicken. The second gel [Fig. 4.41] is from the SB-Whale. The third [Fig. 4.42] is from an extant whale.

Figure 4.43 is the silver-stained gel from fossil sample BFW. Figure 4.44 is the same gel re-stained with Coomassie Blue. Figure 4.45 is a new gel altogether from the same sample BFW sample, stained with just Coomassie Blue.
Figure 4.40 - Gel of the Controls (Cow and Chicken bones), EDTA and GuHCl C₈ elutions.
Figure 4.41 – Coomassie blue stained gel from extant whale. In this gel we assayed a recent (and well preserved) whale bone. The characteristic collagen bands are clearly visible (the two distinct lines on the upper portion of the two central lanes), as well as other bands, which may correspond to other proteins also present.
Figure 4.42 – Silver stained gel from SB-Whale. In this gel we assayed a 20-year old Grey whale skeleton, which had been out under the weather. There are no visible high molecular bands. Its proteins are very fragmented.
Silver Stained gel of sample BFW

Figure 4.43 – Silver Stained gel of sample BFW. There were no visible bands in the gel lanes.

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Figure 4.44 - When re-stained with Coomassie Blue, the gel showed a faint blue line in the bottom of the protein line, but not as far down as the dye front. This can be indicative that the proteins preserved in the fossil are in the form of relatively small peptides.
Figure 4.45 - New attempt to stain proteins extracted from BFW fossil sample, but no success.
Fast Protein Liquid Chromatography

The FPLC result of the “recent whale” control sample appears in Figure 4.46. The fossil sample BFW FPLC is shown in Figure 4.47. A close-up view of fossil BFW FPLC focused at the initial region where higher molecular weight materials would be expected to elute is shown in figure 4.48. We enlarged the results vertically (notice the scale on the left side), in order to be able to see the variance, even if it was very small.
Figure 4.46 - FPLC of an extant whale sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
Figure 4.47 - FPLC chromatogram of sample BFW. The C8 elution fractions of EDTA and GuHCl were combined in order that the signal would be stronger. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
Close-up of FPLC of sample BFW C₈ elution of EDTA + GuHCl combined

Figure 4.48 - This is a close-up of the FPLC chromatogram of sample BFW, when the EDTA and GuHCl supernatants were combined in order to improve the sample's signal. In the close-up, the smaller peaks can be seen, such as the peak between A4 and A7 labeled “A” pointed out by the arrow. It might reflect the presence of the higher molecular weight materials such as Osteocalcin, some larger collagen fragments, or some unknown protein fragment. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
We pooled the BFW FPLC fractions A4-A8 and labeled it “A”, B8-B11 labeled “B”, B12-B14 labeled “C”, and B15-C3 labeled “D”. These fractions were combined as shown in the figure 4.49, and were afterward dried down in order to concentrate them. The letters “A” through “D” correspond to samples later spotted in the MALDI-TOF plate and assayed.

Another earlier extraction [Fig. 4.50] of fossil BFW was also run in FPLC in order to verify the consistency of the results.
Figure 4.49 - FPLC of sample BFW, with EDTA and GuHCl elutions combined. Letters A-D indicate the fractions that were pooled together in order to proceed to the MALDI-TOF mass spectrometry analysis of this sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
Figure 4.50 - An earlier extraction of sample BFW also showing a peak in the beginning of the chromatogram. This peak may indicate the presence of higher molecular weight materials in the fossil sample. Vials A1-C13 are where the FPLC elution solution was collected for further analysis.
Matrix-Assisted Laser Desorption/Ionization-Time of Flight

The blank matrix MALDI-TOF result is shown in Figure 4.51. Control results for chicken [Fig. 4.52-4.53] and cow [Fig. 4.54-4.55] are also shown.

Figure 4.56 displays the MALDI-TOF result for the fossil whale BFW (EDTA extraction). The fossil samples shown in figures 4.57-4.60 are the ones that were subjected to FPLC, which fractions were pooled together.
Figure 4.51 - MALDI-TOF of just the MALDI matrix used in assisting sample ionization.
## MALDI-TOF of: Chicken EDTA Extraction

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**Figure 4.52 - Chicken EDTA 1 MALDI-TOF result. Notice the peak around ~5350 Daltons.**
Figure 4.53 - Chicken GuHCl MALDI-TOF result. Notice the peak around ~5400 Daltons.

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**MALDI-TOF of: Cow EDTA Extraction**

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![Mass Spectrum](image)

Figure 4.54 - Cow EDTA 2 MALDI-TOF result. Notice peak at ~5780 Daltons.
Figure 4.55 - Cow GuHCl MALDI-TOF result. Notice peak at ~5790 Daltons.
Figure 4.56 - MALDI-TOF of BFW EDTA 1, showing some higher molecular weight material around 5400 Daltons.
Figure 4.57 - MALDI-TOF of FPLC Fraction “C” spotting 4 of fossil sample BFW.
Figure 4.58 - MALDI-TOF of FPLC Fraction “C” spotting 5 of fossil sample BFW.
Figure 4.59 - MALDI-TOF of FPLC Fraction “D”, spotting 1 of fossil sample BFW.

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Figure 4.60 - MALDI-TOF of FPLC Fraction “D” spotting 3 of fossil sample BFW.
Dansylation and Thin-Layer Chromatography

The C_{18} column gave a higher DNS-amino acid signal, and a lower DNS-OH signal. Figure 4.61 shows the results of both C\textsubscript{18} and Porapak protocols in the 1\textsuperscript{st} dimension only and Figure 4.62 in the 2\textsuperscript{nd} dimension only.
Figure 4.61 - DNS-Glu polyamide from C18 and Porapak columns (1st dimension only)
Figure 4.62 - DNS-Glu polyamide from C18 and Porapak columns (2nd dimension only)
The results of the controls Gla, Glu, Gla+Glu are shown in Figure 4.63 (the 3 plates run on the 1st dimension). Figure 4.64 compares the 3 plates side-by-side. Figure 4.65 shows the 4 plates run on the 1st dimension. Figure 4.66 compares the 4 plates in parallel. Figure 4.67 shows the Osteocalcin control plate run in 2 dimensions.

Fossil BFW results are shown in Figure 4.68 (1st dimension of BFW and DNS-Cl in parallel). Figure 4.69 shows the TLC of the second dimension (DNS-Cl blank and fossil BFW). A comparison between the fossil BFW plate to the Osteocalcin control plate is shown in Figure 4.70. The distances traveled by the solvent front, when corrected to be relatively equivalent in both the Osteocalcin control and the fossil BFW TLC are shown in figure 4.71.
Figure 4.63 - The 3 plates (Gla, Glu, and Gla+Glu) run in the 1st dimension, and put corner to corner for comparison. The arrows indicate the solvent’s direction.

Figure 4.64 - Movement of the amino acids in the 3 plates (parallel view).
Figure 4.65 - The 4 plates run in the 1st dimension (arrow indicates solvent’s direction).

Figure 4.66 - Movement of the amino acids in the 3 plates (parallel view).
Figure 4.67 - Hydrolyzed and dansylated amino acids from Osteocalcin TLC: 2nd dimension.
Figure 4.68 – 1st dimension of BFW and DNS-Cl TLC. On the left side there is more definition, on the right side we can see the colors better.

Figure 4.69 – TLC of DNS-Cl blank and BFW on the second dimension.
Figure 4.70 - Comparison of the traveled distances between osteocalcin and the fossil sample BFW.
Figure 4.71 - With the adjusted traveled distance, the most prominent spot in the osteocalcin plate seems to be present in the fossil plate and to have traveled about the same distance.
Liquid Chromatography Mass Spectrometry

Figure 4.72 shows the BSA fragmentation pattern. BSA standard sequence matches is shown in figure 4.73. The fossil BFW fragmentation pattern is shown in figure 4.74 and its sequence match in figure 4.75.
Figure 4.72 - BSA LC/MS. The top part represents the full MS pattern, whereas the bottom section represents the fragmentary MS/MS pattern. The peaks from around 70 minutes up unto 100 minutes are very characteristic of peptides.
Mascot Search of LC/MS of BSA Standard

Figure 4.73 - Matching sequences of the standard BSA when compared to a protein database. This standard was digested with trypsin. There are 7 significant protein hits and dozens of matching sequences between this standard and the database.
Figure 4.74 - LC/MS of fossil sample BFW. The upper portion represents the full MS pattern, and the lower portion represents the MS/MS fragmentation pattern. In the same region that peptides appear in non-fossil sample, there is positive signal in the fossil sample. The fragmentation pattern in the lower portion is only due to peptides. If the peaks in fact correspond to peptides, then there are a number of them still present in the sample.
Figure 4.75 - The fossil sample BFW showed no significant hits, when compared to a database that looked for trypsin digested peptidic material. This supports the non-contamination approach used throughout all the course of this study. Because no trypsin was used to digest the fossil sample, trypsin peptides should not appear, except if there was cross-contamination with standards.
CHAPTER FIVE
DISCUSSION

In the microscopy assays of the fossil samples, we observed structures that resembled red colored blood vessels [Fig. 4.01-4.03]. This is suggestive of the possibility that the fossil whales might also have other components preserved, such as high-energy biomolecules (which includes proteins and lipids). In the light microscopy assays, only the fossil sample “BFW” contained structures that resembled the shape of blood vessels.

The SEM results revealed a much higher degree of preservation details than what was seen in light microscopy. It revealed both the vessel-like structures, and also cell-like structures. These cells looked very similar to osteocytes in extant bone. There were also similarities between the fossil vessels and extant vessels:

1. Several of the vessels are branching (as in extant compact bone)
2. The vessels run parallel to the long axis of the bone
3. There are osteocyte-like structures associated with the vessels

Even the fine details present in extant bone such as the canaliculi connecting the osteocyte cells were seen in the fossil. The conditions that allowed for such a high degree of preservation are not yet clear. While the EDTA consumed the fossil bone matrix, it did not consume away the fossil osteocytes.
The XRD results indicate that the fossil blood vessels are replaced by quartz. In the Light Microscopy assay we noted that the vessels would crack when picked up by any metal device. This supports the XRD indication that the vessels are mostly made of quartz.

As for the C\textsubscript{18} extraction of Supernatant 1 being resolved into two phases after centrifugation, if future experimentation confirms the lipid presence, the implication would likely be as surprising as the presence of protein in the fossil samples. Besides lipids being high-energy compounds and especial targets for biomolecular recycling, they are even less likely to be a contamination from microbes than proteins or peptides. Whales are full of lipids in their bones, but bacteria and other microbes simply do not commonly deposit lipids in their environment, which would be too costly.

The UV absorbance of the fossil samples suggests the possibility that the absorbing amino acids are present in the samples. It shows that there is a greater absorbance in extracts of the fossil bone than of adjacent sediment [Fig. 4.23]. This seems to indicate that the fossil bone may have protected its original proteinaceous content better than the soft issues around it did, such as flesh, baleen, skin, etc.

The amount of protein indicated by the BCA assay as being present in the fossil samples vary, but all samples have an overall higher protein amount than the sediment sample (though the sediment sample was taken from a region right adjacent to the bone sample ~ 0.5 cm away from the edge of the bone). Thus, the BCA results indicate what the UV absorption results had already suggested: the presence of small, but detectable amounts of protein in the fossil samples.
Gels from all the control samples showed typical collagen bands (Fig. 4.40-4.41). However, in the gel from the 20 year after death weathering whale, we could not verify the typical collagen bands (Fig. 4.42), not even the least indication of them. There was a lot of lower molecular weight material, which stained very well, but no bands which indicated the complete protein. This may be helpful in understanding how weathered bone shows an immense decrease in original protein content. It might also put a higher cap to the limit of preservation. It seems that, in order for the collagen bands to appear, the whale cannot be exposed to the weather, as was this sample, for several years.

Now when it came to the fossil sample BFW, though we tried several times, we did not see the same bands in the gel [Fig. 4.43], as we saw in the other samples. But the reason for this we understood better when we ran the fossil sample through FPLC, which showed that the sizes of the molecules preserved in the fossils are very small. Both FPLC and MALDI-TOF showed that most of the material preserved weighs from 1000 to 1300 Daltons, which would correspond to an average of 10-13 amino acids long.

When we took this silver-stained gel from fossil BFW and re-stained it with Coomassie blue [Fig. 4.44], we could see something that we didn’t expect. Down below, where the very small peptides would be, we saw a faint blue line, which could be indicative that the material inside the fossil samples is but only a few amino acids long.

We tried running the gel all over again and staining it only with Coomassie Blue [Fig. 4.45], but the results weren’t any more elucidating. There were no bands whatsoever on the upper portion of the gel, which could be because there were no
proteins large enough to be in the upper portion, or because they weren’t enough to show a visible band, or because they didn’t stain.

So we decided that gels were not the most appropriate approach when dealing with fossil material. This was also concluded by Schweitzer as she obtained 8 peptide sequences (Schweitzer et al. 2009) from the dinosaur sample without using gel, but only 1 peptide sequence (described in the Supplement of the same paper) when extracting them from gel. In our study, while gel didn’t seem to give any positive indication that the proteins are present in the fossil, other methods indicated that protein material is there, but we reasoned that they are either too small in size or too little in abundance to stain and be as visible as the proteins from the controls. Fortunately, there were other means of testing if peptides/proteins were present or not in the fossil samples. These other methods were more sensitive, and also relatively more informative. They included FPLC, MALDI-TOF, LCQ, and TLC.

The recent whale doesn’t show discrete peaks as seen in the fossil sample results. This could be because in the recent whale there is a great variety of different peptides, from the breakdown of the larger proteins, resulting in not so discrete peaks. If these peptides are in large concentrations, it would be expected that large peaks would obliterate the display of smaller ones right adjacent to them. Both of these ideas are in agreement with the fact that in recent samples there should be a variety of proteins present, which have not decayed away, but are in the process of breaking down. This process would yield many different fragments of proteins preventing the appearance of discrete peaks.
Fossil sample BFW FPLC results displayed peaks consistent with the presence of peptides (Fig. 4.47). The peaks were right where they were expected in a control sample peptide graph.

Based on this chromatogram and the data obtained from the UV and BCA assays, we may deduce that there are small sized but yet abundant putative peptidic material in the sample. The majority of it is smaller than 2,000 Daltons, and there appears to be discrete peaks, which may suggest that the peptides vary in size. There is enough relative abundance of each type or cluster of peptides so that individual peaks can be distinguished in the FPLC output.

If the sample had anything larger than 7,000 Daltons, it would come out and be chromatographed around vials A4 and A5. That’s where the large molecular weight material should appear. When we take a close-up look (Fig. 4.48) at that region, we see that there is a small, but not random, peak at the end of the void volume. This peak appears between fractions A5–A6. It may reflect the presence of Osteocalcin in the fossil extract or at least some larger molecular weight protein fragments. The relatively large peaks in the regions from B1–C10, may indicate the presence of peptides in various sizes. The putative peptidic presence is in agreement with the BCA assay results.

The fractions that were eluted from the FPLC were combined as shown in the figure 4.49, and were afterward dried down in order to concentrate them. The letters “A” through “D” correspond to samples later spotted in the MALDI-TOF plate and assayed.

Both this BFW extraction [Fig. 4.48] and also an earlier extraction [Fig. 4.50] show a similar peak in the beginning of the chromatogram after the void volume. This
peak may correspond to larger peptides or almost complete small proteins that elute
early following the void volume because of the type of column used.

There is much less material in the region where Osteocalcin or some larger
protein fragments would be than in the region where would be the smaller peptides. This
could mean that the majority of the molecules present in the fossil sample extract are
very fragmented, but that there is a small presence of the higher molecular weight
material. Regardless of peak sizes, the FPLC results are consistent with the presence of
peptides in the fossil sample.

The MALDI-TOF results were much more elucidative than any other type of
experiment we had previously done. There was indication of higher molecular weight
materials in the fossil samples, such as Osteocalcin, which has a molecular weight
~5800 Daltons in extant samples. The chicken [Fig. 4.51-4.52] and cow [Fig. 4.53-4.54]
controls showed peaks at similar ranges where one would expect Osteocalcin to show
up. The cow peaks are closer to the ~5800 Daltons commonly attributed to Osteocalcin
than the chicken. However, even if they are not exactly alike, the pattern seen in the cow
peaks is similar to the pattern in the chicken peaks. This could be some size variation in
this protein across species, as seen in our samples.

Something to consider in the fossil sample is that the C₈ EDTA elution of the
sample “BFW” [Fig. 4.56] displayed a peak at ~5400 Daltons, which possibly suggests
that remnants of the protein Osteocalcin may still be present in the fossil sample, as
there have been previous reports of Osteocalcin in fossils (Ulrich et al. 1987; Huq et al.
1990; Muyzer et al. 1992; Collins et al. 2000; Nielsen-Marsh et al. 2002). One thing to
note though is that we were only able to see this in one extraction of sample “BFW”
using MALDI-TOF, and possibly in two other extractions using the FPLC column [Fig. 4.48 and 4.50].

The reason for this in the case of MALDI-TOF may be because the extraction solution we used when the peak showed up was not concentrated, but was just eluted out of the C₈ column, whereas all the other times when we tested the extraction with MALDI-TOF, the solutions had been previously concentrated down using a laminar flow of nitrogen. This might have caused the proteins to clump together and, due to its specific chemical properties, come out of solution.

Another consideration in the fossil sample is that it could be composed of very fragmented proteinaceous materials, which would not be seen in the higher range of the MALDI-TOF, but only in the peptide range. In accordance with that, we point out that when we used the peptide method (instead of the protein method) to analyze the fossil elution, we saw peaks consistent with the FPLC results. The peaks presented very consistent sizes (which were absent in the blank results, i.e. they were extra peaks found in the fossil sample BFW).

We see in the plain matrix or “the blank” [Fig. 4.51], which is used in assisting the sample molecules to be ionized and fly off the plate, its characteristic peaks. These peaks are related to the sizes of the materials composing the matrix solution and their interaction with each other. The largest considerable peaks (visible as a peak and not just as a number in the list of numbers) range up to ~909 Daltons.

Now compare this to the fossil peaks [Fig. 4.57-4.60]. Clearly there are indications that the fossil has some extra peaks (corresponding to molecules that are present in the sample) aside from those present in the matrix. These fossil samples are
the ones that were subjected to FPLC analysis, which had their fractions were pooled together.

Some peaks shown in the MALDI-TOF output, but absent in the matrix include: 927, 1033, 1052, 1077, 1096, 1106, 1161, 1287, 1310, 1349, 1498, and 1538 Daltons. These are not including anything below the 908 Daltons (highest blank matrix peak shown seen in Fig. 4.51). There are slight variations in the fossil peaks (1-2 Daltons), but they seem to be quite consistent throughout the analysis. The identification of peaks that are not in the blank but that are in the fossil is consistent with the presence of peptides in the fossil sample BFW.

MALDI-TOF seems to be supportive of the presence of small and fragmented peptides in the fossil sample BFW, besides those that are present in the blank.

The dansylation protocol was only partially elucidative. The first point we saw was that instead of using Porapak columns for the procedure, we had better preliminary results with the C18 columns. The C18 column gave a higher DNS-amino acid signal (what we want to see), and a lower DNS-OH signal (what we want to get rid of) [Fig. 4.61 and 4.62].

Running the experiment with C18 column using the amino acid retrieval protocol is more efficient for removing the DNS-OH and keeping the DNS-Glu than the Porapak column procedure.

When the DNS-Cl (the blank) is compared to the fossil sample BFW [Fig. 4.69], it is obvious that the fossil has some extra material than the blank. If we compare the BFW plate to the Osteocalcin plate [Fig. 4.70] there are some interesting similarities and also differences. The differences appear in the number of visible spots. The Osteocalcin
control has about a dozen visible marks, whereas the fossil BFW sample only shows 4-5 spots. This could be due to the fact that the fossil has been decaying and not all amino acids are present in enough amounts to result in a visible spot for each one. The fossil may or may not have all the amino acids, but because they might be present in such low quantities, it may not be possible to detect them using this method.

An important similarity though is evident when we digitally correct the solvent’s front traveled distance to match between both the control and the fossil sample plates [Fig. 4.71]. The distance traveled by the solvent front can be affected by external factors, and not by the sample alone. There is a ratio between the traveled distance of the solvent front and the traveled distance of each spot. However, we can digitally correct the traveled fronts by stretching the image, and the spots behave as if they had traveled accordingly as well. If we digitally adjust the solvent’s traveled distance to account for the smaller total travel length in the Osteocalcin TLC as relative to the fossil TLC, the amino acid in highest abundance in the Osteocalcin standard is located right where is located the only one visible in the fossil TLC. Their most prominent spots seem to have traveled similar distances. The spot in the fossil plate is almost the only one seen in the plate (except for the spot of unknown composition located at the top-left corner). If the brightest spot in the Osteocalcin control plate corresponds to the brightest spot in the fossil BFW plate, then, it is possible that the fossil didn’t have enough material to display all amino acids such as the ones shown in the control, but only the most prominent one, and the others are too faint to be noticed in this assay.

The support of the TLC assay for the presence of amino acids in the fossil samples is not as evident and straightforward as are the previously reported assays.
Nevertheless, it still offers some level of support as shown by the equivalent distance traveled by the most prominent spots.

The LC/MS results are very extensive, and the data technically demanding. In a trial run of the DE NOVO sequencing there were over 11,000 entries or putative amino acid sequences found in the fossil sample BFW LC/MS. The confidence level of the majority of those fragments is not high, but there are some, which present higher degrees of confidence of yielded sequence.

The process of analyzing those sequences can be extremely time-consuming, and demands an expertise that is not readily available. This particular step may require considerable more effort than initially thought, rendering it more appropriate for a doctoral dissertation, or a multidisciplinary approach, that is, mass spectrometry trained people in conjunction with biochemistry and paleontology.

Nevertheless, we included in the results the overall fragmentation pattern obtained from both the BSA standard and from the fossil sample BFW. When the BSA LC/MS output result was searched at the protein database (Mascot, from Matrix Science), there were over 7 protein hits, with dozens of matching sequences [Fig. 4.72], indicating that the system correctly identified the LC/MS results for the BSA standard. The search was performed by selecting trypsin as the enzyme responsible for the digestion of the BSA protein. The fragmentation pattern seen in the secondary ms/ms is characteristic of peptidic material (lower half of Fig. 4.73).

When we were performing the search with the fossil sample BFW LC/MS results, we realized that we did not have an option for choosing “no enzyme” in the search system. Although the fossil was fragmented, the fragmentation process was not
specifically done by trypsin or any other specific enzyme, but by means of random
to processes, of which we have no current knowledge. As we performed the search (using
trypsin as the enzyme responsible for digestion), there were absolutely no significant
hits, with zero matching sequences to anything, implying that the system did not identify
any peptide fragments that were cleaved by trypsin.

This result makes sense because the search was only looking for trypsin digested
peptides. It is evident, therefore, that there was no cross contamination between the BSA
standard and the fossil sample, for if there were, the trypsin digested fragments would be
pointed out. The implication is that, whatever we find in the fossil sample BFW LC/MS
output when trying to reconstruct the peptide fragments by means of the DE NOVO
sequencing belongs to the fossil, or at least, is not a contamination from the standard.

The fragmentation pattern of the fossil sample BFW (Fig. 4.74) is happening in
the same region that it usually happens when extant proteins are analyzed. Secondary
fragmentation is happening, which indicates that the material we are dealing with is
composed of peptides. This is very important in determining the nature of the material
from fossil BFW, which has already been indicated in the previous assays such as BCA,
FPLC and MALDI-TOF. The sum of factors suggests that the material consists of
peptides/proteins and actually behave as that. The LC/MS assay was a further
confirmation of the previous steps in the analysis of fossil sample BFW.

As to which peptides or sequences they specifically represent, may be a question
more appropriate for a more in-depth study, and not feasible for a masters program.
CHAPTER SIX
CONCLUSION

The fossil whales of the Pisco Formation are of import, not only from an inorganic, sedimentary perspective, but also from the organic, molecular, and ultrastructural perspective. In addition to still articulated bones and the presence of fossil baleen in life position, the results of the experiments in this current study suggest that there is also a certain level of biomolecular preservation.

Because of the altered nature of animal remains found in the fossil record, they are far more complex to analyze than extant samples. There is potential for a lot of change to happen in the course of time of which we may have no idea. Even though we can do repeated experiments, it is not with absolute certainty that conclusions can be drawn. Nevertheless, some conclusion may prove of value.

One of these conclusions is that when working with fossil candidates for extensive experimentation, screening them with even a light microscope may prove to be very timesaving (the promising samples may be more easily indicated). In this study, the microscopy assay showed that blood vessel structures and osteocyte shapes were still present in some of the samples. The composition of the vessels was indicated by XRD to be quartz. This may imply that silica-rich fluids have passed through the sample and precipitated. The extent of this silica deposition is not complete, for there is still some organic material present in the samples, as indicated by the protein/peptide assays.
The preservation of fossil osteocytes is also of relevance, especially because of the abundance seen in these samples. Just like the fossil blood vessel structure, these osteocytes were also not consumed when subjected to EDTA. Both may have the same constitution, or at least, may have been partially substituted by the same minerals. If the samples had been in an aqueous environment too long, these structures could have been decomposed, and the fine details could have been lost (Allison et al. 1991; Brand et al. 2003; Trueman et al. 2003).

Unlike the surprising T-rex sample (Schweitzer et al. 2005), which contained still soft and pliable original tissue, our samples, after partial demineralization remained brittle, implying that much of the organic material had been substituted by mineral components. In spite of that, we still observed well-preserved morphological features and evidence of proteinaceous, as well as lipid, material.

The level of proteinaceous preservation in the fossil samples analyzed in this study varied considerably. Nevertheless, it is worthy of notice that when fossil sediment was compared to fossil bone, even though the sediment sample was located immediately adjacent to the fossil bone, it presented an average protein concentration below that present in all fossil bones analyzed, including those badly preserved, implying that the fossil bone provided a better environment for biomolecular preservation than its surroundings. The detection of preserved proteinaceous material from the fossil bone samples is supported by the BCA assay, the TLC analysis, the FPLC profile, the MALDI-TOF mass spectra, and the LC/MS results (especially the secondary fragmentation pattern characteristically observed in peptidic material). In addition to extracted proteinaceous material, there was also indication of lipid preservation in the
C$_{18}$ extraction of the EDTA supernatant from the fossil sample BFW. This is consistent with a number of reported lipid findings in fossil samples (Das et al. 1967; Mackenzie et al. 1982; Eglinton et al. 1991; Briggs et al. 2000).

We conclude that this study contributed to the growing body of knowledge of the fossil whales from the Pisco Formation and of the extent of preservation found in them. Although the previously assigned geologic ages to these whales did not encourage searching for biomolecules in them, the actual results of these experiments are supportive of the presence of peptides/protein fragments in these fossil whales. Much more research can and should be done to determine with greater depth the extent of preservation of both the ultrastructure and the organic remains of the fossil whales from the Pisco Formation.


