学位論文 （要約）

# Application of new analytical method in biological archaeology: proteomics of archaeological human bones and DNA analysis of dental calculus

# (生物考古学における新たな分析手法の応用: 古人骨プロテオミクス解析と歯石 DNA 分析)

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# Application of new analytical method in biological archaeology: proteomics of archaeological human bones and DNA analysis of dental calculus

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# Abstract

Over the last four decades, biological archaeology (bioarchaeology) has achieved remarkable growth. Some advances have emerged from improvements in methods and new technologies. On the other hand, problems that can not be clarified by the limitation of conventional bioarchaeological methods still remain. By acquiring information that could not be obtained by conventional methods, it is possible to investigate the past human life from a new viewpoint. The purpose of this study is to apply two new methods to bioarchaeological research.

The first is shotgun proteomics analysis of ancient human bones. Ancient protein analysis provides clues to human life and diseases of ancient times. While immunological methods have been used, the reliability of these methods remains uncertain and cannot be simultaneously applied to multiple proteins. I performed shotgun proteomics of human remains using eight rib bones excavated from the Hitotsubashi site of the Edo period, expecting to acquire physiological information. The output data obtained were analyzed using Gene Ontology and label-free quantification. As a result, a total of 668 proteins were identified, and not only collagen but also various kinds of proteins such as extracellular matrix were identified. I detected leukocyte-derived proteins, possibly originating from the bone marrow of the rib. Particularly prevalent and relatively high expression of eosinophil peroxidase suggests the influence of infectious diseases at that time. This scenario is plausible, considering the overcrowding

and unhygienic living conditions of the Edo city described in the historical literature. I also observed age-dependent differences in proteome profiles, particularly for proteins involved in developmental processes. Among them, alpha-2-HS-glycoprotein (AHSG) demonstrated a strong negative correlation with age. There is a possibility that age information is estimated using the expression level of proteins correlated with age such as AHSG.

The second is food DNA analysis from dental calculus. Isotopic analysis and starch grain analysis have been used as methods of reconstructing ancient diet. The limitation of these methods is the difficulty of species/genus identification. Moreover, organs such as leaves, stems, and roots are soft and hard to remain at a site, which prevents the reconstruction of diet inclusively. In order to investigate diet diversity of the past, calculus samples of human remains excavated from the Unko-in site in the Edo period were collected. PCR amplification and sequencing were performed using the genus *Oryza* specific primer set, because rice (*Oryza sativa*) is the staple food and the only genus present in Japan at that time. The sequence was detected from more than half of the individuals, and this result indicates that ancient calculus contains food DNA. Furthermore, in order to know dietary diversity, DNA metabarcoding targeting plants, animals (meat and fish) and fungi was carried out using eight individuals which showed the genus *Oryza* DNA amplification. As a result, seven families and ten genera of plants were detected in total. Most of the taxonomic groups are present in Japan, but the only one that does not exist is the family *Dipterocarpaceae* inhabiting the tropical lowlands.

At that time, borneol (*ryunou* in Japanese) derived from *Dryobalanops aromatica* was used as a component of tooth powder, so the taxon is likely to be derived from the debris of tooth powder. Some fungi were also detected from the calculus, despite animals except for human were not detected. From these results it is suggested that analysis of dental calculus provides information about food diversity and lifestyle habits of the past.

These methods offer new insights into bioarchaeology and complement conventional methods to reconstruct human life of the past more comprehensively.

# Contents

# 1 General Introduction

## 1.1 Methods of biological archaeology

Biological archaeology (bioarchaeology) is the scientific study of biological remains from archaeological sites. This field began just four decades ago (Buikstra, 1977), by combining elements of physical anthropology and archaeology (Klaus, 2014; Stojanowski and Duncan, 2015). Now bioarchaeology relies on interdisciplinary research. It is related to anthropology, archaeology, ethnology, history, geography, geology, physics, chemistry, paleoecology, paleontology, paleozoology and paleobotany. Thus, the field has incorporated methods of other fields and evolved. For example, stable isotope analysis has been used extensively in the field of geology and ecology, and it applied to archaeological bones for investigating past diet since the 1970–1980s (Katzenberg, 2008). As the field matures, associated fields have developed such as reconstruction of breastfeeding and weaning practices (Tsutaya and Yoneda, 2015). The emergence of next generation sequencing (NGS) technologies has also enabled ancient DNA studies to evolving into a powerful research tool. This field has entered the new era of genomics and has provided valuable information about past human history (Der Sarkissian et al., 2015). It has enabled the analysis of the genomes of Neanderthals, Denisovan, and Middle Pleistocene hominoids (Meyer et al., 2016, 2012; Prüfer et al., 2014). In recent years, it has also accelerated ancient population analysis, dealing with more than one hundred individuals (Haak et al., 2015; Lazaridis et al., 2016). These results provided

information of the evolutionary and the migration history of humans in detail.

### 1.1.1 Proteomic Research

In recent years, not only paleogenomics but also paleoproteomics attracts much attention. Lots of questions that analyses of ancient DNA cannot answer have left behind so far, which makes it difficult to obtain the full picture regarding human life of the past. Now thanks to remarkable improvement of the mass spectrometry (MS) and protein databases, ancient protein has been gradually giving the clue of these questions. For example, proteins are more prone to be preserved than DNA (Wang et al., 2012), so they could be extended further back in time. Although the time depth of ancient DNA analyses is ∼400,000 years in temperate areas and ∼700,000 years in permafrost (Dabney et al., 2013a; Meyer et al., 2014; Orlando et al., 2013), ancient proteins from bones sometimes remain over one hundred million years (Schweitzer et al., 2014). Species identification and phylogenetic relationships based on the protein analysis are sometimes more preferable than that by DNA analysis due to this reason (Buckley and Wadsworth, 2014; Welker et al., 2015). Furthermore, protein expression is specific to different tissues, developmental phases, disease or biological processes (Cappellini et al., 2014a). Taking advantage of the features, several studies revealed human diseases of the past. Shotgun proteomics applied to human medieval dental calculus identified bacterial virulence factors and host immune defense proteins (Warinner et al., 2014b). Mummified remains are well-used samples for detecting a host immune system response

to severe bacterial infection (Corthals et al., 2012; Hendy et al., 2016; Maixner et al., 2016). Detecting disease-associated proteins from human bone is promising for contributing to paleopathology, too (Bona et al., 2014; Boros-Major et al., 2011; Kendall et al., 2016).

### 1.1.2 Ancient plant DNA

The analyses of past diet are often difficult. Especially the consumption of plant foods is difficult to analyze because plants are hardly remained at a site. Most plant materials are subject to rapid decomposition and thus the majority of archaeological plant materials is preserved in a charred state (Nistelberger et al., 2016). This prohibits the reconstruction of diet comprehensively. For example, Habu (2015) discussed the mechanisms of long-term changes in ecosystems and subsistence-settlement systems (economic and social systems) from the perspective of historical ecology and resilience theory. Because of the difficulty of evaluating food diversity directly, the author used lithic assemblage diversity as a substitute for food and subsistence diversity. Thus, food diversity is the capital information for reconstruction of human behavior of the past although the method of analysis is undeveloped.

### 1.1.3 Objectives of this study

In this study, I focused on applying new methods to bioarchaeology for overcoming these challenges and shedding light on the critical piece of human behavior. In chapter

2, I applied shotgun proteomics analysis for ancient human bones for the first time. The analysis revealed that levels of some proteins existed in bones are correlated with age at death of individuals and that traces of past immune responses remained in bones. In chapter 3, I applied DNA metabarcoding analysis of plants for ancient human calculi for the first time. The analysis demonstrated that the information of ancient food taxa at the genus levels could be extracted from calculus samples and also revealed a past human habit such as brushing teeth. In chapter 4, I explained the achievement, comparison with conventional methods, limitations, and prospective of these two methods.

# 2 Proteomic profiling of archaeological human bone

本章については、Royal Society Open Science で公表前のため、非公開。

# 3 Ancient calculus DNA analysis of foods

本章については、5 年以内に雑誌等で刊行予定のため、非公開。

# 4 General Discussion

## 4.1 Proteomic profiling of archaeological human bone

### 4.1.1 Advantages

Proteomic analysis has four advantages when compared with DNA analysis.

a) Longer preservation. Ancient DNA can be analyzed up to ∼700,000 years old at most, in case of permafrost (Orlando et al., 2013). Ancient DNA is susceptible to fragmentation and chemical modification, and the state of preservation of the sample depends greatly on the place that the sample excavated and stored. It is well preserved in a dry and cold climate like Europe, and this allows determination of Neanderthal genomes (Noonan et al., 2006). Meanwhile it is extremely difficult to analyze ancient DNA of remains in acidic soil environment with humidity like in Japan, even if the sample is only thousands of years old.

However, it has been demonstrated in several studies that it will be traced back to at least over a million years ago in regard to ancient protein (Buckley and Wadsworth, 2014; Demarchi et al., 2016; Schweitzer et al., 2014). There is a case that ancient DNA could not be analyzed but ancient protein could be analyzed from the same sample (Welker et al., 2015). These suggests that ancient proteins tend to preserve in bones longer than ancient DNA. Several reasons for longer preservation of ancient proteins are proposed in previous studies. In regard to collagen, a straightforward explanation

is rich content (Collins et al., 2002) and limited solubility (Perumal et al., 2008; Trueman and Martill, 2002), and both provide excellent prerequisites for the survival of the type I collagen. Wang et al. (2012) reported that the elevated abundance of thermostable amino acid residues in type I collagens contributes to its survival. Demarchi et al. (2016) proposed other hypothesis that peptides which has the domain with the strongest calculated binding energy to the mineral surface is selectively preserved in bone.

The reason that I expected to be one of the cause of ancient protein superiority is that ancient protein identification is easier than ancient DNA alignment, owing to the variety of amino acid type. In other words, proteins can be identified by shorter sequence than DNA because of the rich variety of units that make up the sequence. Average molecular weight of an amino acid is 110 daltons, and that of single-strand DNA base is 330 daltons. More than five peptides are commonly used as the minimum peptide length for identifying proteins (Kim et al., 2014; Marcus, 2012), which is too short for DNA to be identified its species and regions. There are $6.4 \times 10^7$ types for six peptides ideally, which corresponds to 12 bp. Determination of gene or taxon from 12 bp fragment of DNA is absolutely impossible. This is a simple estimation and energy calculation of organic chemistry is needed, but this character may be one of the reason for easy identification of proteins from older samples.

b) Minimum sample requirement. Molecular biology experiments are basically crushing experiments. Therefore, it is an advantage to be able to analyze with as

small samples as possible. When using bones or teeth as samples, approximately 200 mg to 3 g is necessary for DNA analysis. On the other hand, in the case of proteins, samples can be analyzed if they are approximately ∼40 mg. This enables the protein analysis of even only a small amount of deposits of pottery (Solazzo et al., 2008).

c) Lower contamination. Contamination of modern DNA is a serious problem when analyzing ancient DNA, and it is necessary to use special equipment which prevent contamination from the outside. On the other hand, in the case of ancient proteins, contamination is unlikely to strongly impact on the analysis compared with DNA. This seems to be mainly because of three reasons; (1) more residual amount, (2) no amplification process like PCR (This process can skew the original abundance ratio), and (3) tissue-specific expression pattern which makes it easy to discriminate contaminating proteins (e.g., keratin of skin).

d) Information not available in DNA. Basically, genome is identical in every somatic cell in our bodies. However, the expression pattern of proteins varies between tissues and cells. For example, when analyzing dental calculus, it is impossible to distinguish between beef and milk by DNA analysis, albeit possible by protein analysis. Thus, ancient protein analysis is quite effective in investigating human activities such as the origins of dairy farming (Warinner et al., 2014a).

### 4.1.2 Limitations

As a disadvantage of ancient protein analysis, there are few differences of sequences between species and among individuals, due to its codon degeneracy. This means that protein analysis is not appropriate for species identification or screening genetic information for each individual. There is also a possibility that the results of protein analysis may be affected by the extraction method (Jiang et al., 2007). Considerable caution is needed when comparing samples extracted by different methods.

### 4.1.3 Perspectives

It is conceivable that physiological information of individuals such as pregnancy and disease could be obtained from analyzing ancient bone proteins. In this study, it was revealed that various kinds of blood proteins were present in ancient bones. This indicates that not only proteins expressed in bone but also proteins expressed in blood can be detected from ancient bones.

Pregnancy-associated plasma protein-A (PAPP-A) is one of proteins of placental origin found in high concentrations in the blood of pregnant women (Boldt and Conover, 2007). Human placental lactogen (hPL) and human chorionic gonadotropin (hCG) also have essential roles in pregnancy and expressed in the blood. Detecting these proteins from ancient bone will lead to the pregnancy status of past human populations.

As for disease, Schultz et al. (2007) reported that they detected PSA (prostate specific antigen), which is an important marker for the diagnosis of prostate cancer. Cancer of the prostate gland, lung, breast, kidney, stomach, thyroid gland as well as multiple myeloma frequently metastasize to bone. In the case of those diseases which metastasize to bone and diseases which change the state of blood, there is a possibility of revealing the disease by analyzing proteins within bone.

## 4.2 Ancient calculus DNA analysis of foods

### 4.2.1 Advantages

The advantage of food analysis of calculus is genus/species level identification. Species-level identification was not tested in this study, but it is possible in theory. This method also enables analysis of organs hardly remained at the site (e.g., leaves, roots, and rhizomes). This method is complement to other methods such as isotope and starch analyses.

In regard to the merit of metabarcoding, it is more cost-effective and suit for population analysis. It is also compatible with screening food DNA at that time. If an interesting taxon is detected by this method, one can expand more specific research, for example, examining nuclear genes or correlation with oral microbiota.

### 4.2.2 Limitations

The most important problem is contamination with modern DNA, especially soil DNA. There is a possibility that some ancient calculus DNA was contaminated by soil. It is difficult to exclude totally the effect of soil DNA, but there are some solutions for decreasing it.

a) Excluding soil from calculus surface as much as possible. Sometimes the surface of calculus was covered with soil. This should be excluded thoroughly by brushing, scraping, or washing. As for ancient bones and teeth, the outer surface of the samples was often removed using a dental drill (Adachi et al., 2009), but applying this step to the calculus samples is not so easy. The use of only inner calculus (the adjacent part to the teeth) may be desirable, but calculus tends to be very thin, small and brittle. So, UV irradiation of the surface is more practical step for calculus analysis. Some previous studies used EDTA as a decontamination step (Ozga et al., 2016; Warinner et al., 2014b). Others even used bleach for decontamination (Warinner et al., 2014b; Weyrich et al., 2017). However, artificial depurination can be caused by bleach treatment used for decontamination (García-Garcerà et al., 2011). Depurination is a key component of post-mortem fragmentation of ancient DNA molecules (Dabney et al., 2013b; Hofreiter et al., 2001), so one must pay serious attention to it when using bleach for decontamination, if there is need for checking ancient DNA authenticity (Kihana et al., 2013).

b) Comparing with soil as much as possible. Soil sampling from mandibular foramen was not so much, about 20 mg from each foramen. It is good if one can participate in excavation for sampling a large amount of soil. Improving DNA extraction methods from soil is also desirable, as impure substances in soil may inhibit enzyme reaction. In the field of ecology, Extraction kits of MO BIO Laboratories, Inc. are often used for soil samples (Epp et al., 2012; Young et al., 2014).

c) Population analysis. Population analysis is also pivotal for the confidence of identified taxon. The more samples one uses, the more reliable the results are. Ideally, it is better to show that there is a significant difference in regard to certain taxon by comparing the calculus samples and the soil samples.

d) Combination with other methodology. Combining with other methodology such as starch grain analysis and proteomic analysis increases the confidence of existence of identified taxon. This may be efficient especially when analyzing staple foods. Performing PCR with species-specific primer will also efficient for checking whether the identified plant DNA is come from food species or weed.

In the field of starch analysis of calculus, the cases of screening both calculus and soil samples are really rare. Starches in sediments are not analyzed in most starch analysis of calculus (Henry and Piperno, 2008; Piperno and Dillehay, 2008; Wesolowski et al., 2010). This is a serious matter because starch granules basically remain in soil environment (Shibutani et al., 2015). Shibutani (2015) pointed out that it is necessary to collect and analyze the surrounding soil when analyzing starches from

stone tools for checking contamination, although no one depicts the risk of starch analysis from calculus samples without soil samples. As for ancient plant DNA analysis, it is important to examine the sample of soil too.

The other point of limitations is that this method is difficult to estimate the quantity of the food consumption. From the results of starch grain analysis and isotopic analysis, it has been pointed out that calculus may not reflect much of the intake of food. In the starch grain analysis, it was investigated the relationship between plant microremains (the number of starches and phytoliths) in calculus and plant consumption of modern hunter gatherers and chimpanzee populations. They found that starches and phytoliths do record diet, but that the relationship between diet and microremains is not as straightforward (Leonard et al., 2015; Power et al., 2015). According to a study comparing isotopic analysis of bone and calculus, the first attempt is to investigate the potential of calculus as a substitution of bone for reconstructing diet (Scott and Poulson, 2012). However it was revealed that the carbon and nitrogen isotope values of bone and calculus do not correlate, or have a weak correlation (Eerkens et al., 2014; Salazar-García et al., 2014). From these results, it is better to assume that the food residue in the dental calculus preserved by chance rather than cumulatively.

### 4.2.3 Perspectives

By using this method, diversity of food in the times without literature can be investigated. For example, it is an important issue that whether people during the Jomon

period consumed yams such as *Dioscorea japonica* and *Dioscorea polystachya* commonly. The spread of rice in the Yayoi period and the possibility of eating millet are also issues that ancient calculus could reveal.

It is also a good point of this method to be able to investigate the existence of trade. In this study, the possibility of trade was presented from plants of the family *Dipterocarpaceae*. For this purpose, it is necessary to eliminate and decrease contamination of modern DNA as much as possible to increase certainty.

It is also interesting to analyze the habits of the oral hygiene. Although I discussed the use of tooth powder this time, in the previous study the use of medicinal herbs has been discussed in starch grain, chemical analysis of calculus (Buckley et al., 2014; Hardy et al., 2012). Combining multiple methods for calculus analysis lead to unveiling the past human life more deeply and certainly.

There is room for improvement of the method. Analyzing with DNA hybridization capture instead of with PCR for DNA metabarcoding enables to reduce the effect of contamination and to detect deamination which is the authenticity of ancient DNA. It also allows the analysis of nuclear genes involved in domestication such as shattering resistance. Nistelberger et al. (2016) reported that high-throughput sequencing technologies are not suitable for use with charred archaeobotanicals. There is a possibility that the genome can be analyzed from the food debris of calculus which is not charred.

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