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Effects of lipid extraction and different collagen extraction methods on archaeological fish bones and its implications for fish bone diagenesis

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A B S T R A C T

Gelatin extracted from archaeological fish bones typically exhibits relatively high C/N ratios, presumed to be caused by contamination with lipids or humic substances. The effects of lipid extraction and different collagen extraction methods applied has been studied on modern fish bones but has never been studied systematically on archaeological specimens because of taphonomic and experimental reasons. In this study, the effects of lipid extraction and order of NaOH treatment in collagen extraction method on carbon and nitrogen stable isotope analysis of archaeological fish bone (n = 30) excavated from the Hamanaka 2 site, Hokkaido, Japan (approximately 8th BC–10th AD) is investigated. Gelatin extracted from the same fish bone subsamples with or without lipid extraction procedure indicates neither significant nor systematic differences in carbon and nitrogen stable isotope ratios, elemental concentrations, C/N ratios, and yield. However, reproducibility of stable isotope ratios and elemental concentrations decrease in gelatin extracted from poorly-preserved cod bones (< 3.5% yield). Gelatin extracted from archaeological fish bones may contain humic contaminants, and its effect becomes greater in gelatin with lower extraction yield. Although there is no significant change in the stable isotope and C/N ratios, change in atomic concentration of carbon and nitrogen suggests that the purity of extracted gelatin increases when NaOH treatment is applied after decalcification. Because this is only a study from one archaeological site, further case studies that evaluate lipids and diagenesis in fish bones are required.

1. Introduction

Although carbon and nitrogen stable isotope analysis of collagen extracted from ancient fish bones is an important research topic in (bio) archaeology (e.g., Barrett et al., 2008; Guiry et al., 2016a; Szpak et al., 2013), there is less technical consideration on the collagen extraction procedures compared with those for ancient mammalian bones (e.g., Guiry et al., 2016b; Nicholson, 1996a, b). Preservation and yield of collagen in fish bones are generally poor compared with that in mammalian bones (Nicholson, 1996a, b; Szpak, 2011). Degradation of collagen and contamination by non-collagenous molecules are the probable causes of the poor status of fish bone collagen. The compact packaging of collagen, protected by the mineral phase of bone, is important for the preservation of collagen (Collins et al., 2002). However, fish bones contain less mineral phase and consist of more loosely-mineralized collagen than mammalian bones (Lee and Glimcher, 1991), which would lead a greater degradation (i.e., biotic attack) of fish bone collagen in the burial environment (Szpak, 2011). Humic substances, the major component of soil contaminant (van Klinken and Hedges, 1995), would easily penetrate loosely-mineralized fish bones and interact with collagen (Szpak, 2011). Furthermore, fish bones generally contain higher amounts of lipids than mammalian bone (e.g., Toppe et al., 2007), and the residual lipid might contaminate extracted gelatin (Szpak, 2011). Therefore, it is important to evaluate the validity and utility of various procedures of collagen extraction in ancient fish bones.

Lipid extraction procedures are important to measure the stable isotope ratios of proteinous tissues (e.g., Logan et al., 2008; Post et al.,

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2007; Sweeting et al., 2006). This is because lipids contain large amounts of carbon but little nitrogen and typically present lower stable carbon isotope ratios (δ^{13}C value) than most proteins (Ambrose, 1990; DeNiro and Epstein, 1977). Therefore, lipid contamination in extracted gelatin results in elevated C/N ratios and lower δ^{13}C values. Szpak (2011) indicated that gelatin extracted from archaeological fish bones typically exhibits relatively higher atomic C/N ratios than modern ones. While the calculated C/N ratios of bone collagen based on amino acid compositions for ray-finned fishes, Actinopterygii (3.15 ± 0.04, n = 12), are statistically significantly lower than those for Mammalia (3.23 ± 0.04, n = 23), measured actual C/N ratios for gelatin extracted from archaeological bones of Actinopterygii (3.38 ± 0.52, n = 1127) are statistically significantly greater than those of Mammalia (3.28 ± 0.33, n = 1836) (Szpak, 2011). The difference still remains even if the only gelatin extracted with ultrafiltration is considered (Szpak, 2011). Contamination by lipid is hypothesized to be one of the possible causes of this higher ratio, as well as contamination with humic substances (Szpak, 2011).

However, there has been little study to evaluate the effect of lipid extraction and different extraction methods on archaeological fish bones (but see Guiry et al., 2016b for modern fish bones). This is because: i) the scattered and relatively small size of archaeological fish bones prevent making the replicate measurements required for comparison of different extraction methods; ii) evaluating the effect of lipid extraction *per se* is difficult because the treatment by alkali solution (e.g., NaOH) of bones is essential when extracting collagen from archaeological samples but works like lipid extraction except for phospholipids (Ambrose, 1990); and iii) there is no significant effect of contaminated lipid on the isotope ratios in fish bones with initial lipid concentration of less than 5% (Guiry et al., 2016b), and these species should be omitted for the evaluation of lipid extraction on archaeological fish bones. Different collagen extraction methods produce little systematic difference in archaeological bones with higher collagen yield, but bones with lower collagen yield tend to show inconsistent isotope ratios and elemental concentrations when applying different extraction methods (Dobberstein et al., 2009; Pestle, 2010; Sealy et al., 2014; Tuross, 2012). Fish bones with lower collagen yield would be of special interest for evaluating the isotopic and elemental consistency between different extraction methods.

In this study, the effect of lipid extraction on carbon and nitrogen stable isotope analysis of archaeological fish bone gelatin is investigated. Also, we compared different collagen extraction methods for poorly-preserved fish bones. The following topics are investigated for the extracted gelatin from archaeological fish bones.

- Contamination by lipids: If lipids contaminate gelatin extracted from archaeological fish bones, it is expected that gelatin extracted without lipid extraction will present: i) increased yield because of the presence of lipid in the extracted gelatin; ii) increased atomic concentration in carbon (%C) and C/N ratio because of the high number of carbon atoms in lipid and the absence of nitrogen; and iii) decreased δ^{13}C values because of the typically lower δ^{13}C values of lipid compared to collagen (Ambrose, 1990; DeNiro and Epstein, 1977), as indicated in the study of modern fish bones (Guiry et al., 2016b).
- Unexpected effect of lipid extraction: Although there is no systematic difference in δ^{15}N values of gelatin extracted from modern fish bones with and without lipid extraction (Guiry et al., 2016b), it is possible that δ^{15}N values of tissues alter unexpectedly after the application of lipid extraction possibly because of incidental leaching of tissue proteins (Logan et al., 2008; Sotiropoulos and Tonn, 2004). Collagen in archaeological bones is degraded and contaminated to a certain amount, and needs to be evaluated separately from modern specimens.
- Order of NaOH treatment: Most collagen extraction methods for stable isotope analysis adopt NaOH treatment after decalcification (e.g., Pestle and Colvard, 2012; Sealy et al., 2014; Szpak et al., 2017), but the NaOH treatment is applied before decalcification in some studies (e.g., Yoneda et al., 2004; Tsutaya et al., 2017). Although humic substances are also removed when applying the NaOH treatment before decalcification in well-preserved bones (Yoneda et al., 2004), the efficiency of the NaOH treatment before decalcification in poorly-preserved bones is unclear. Therefore, it is important to evaluate the isotopic consistency between extracted gelatin with the NaOH treatment before or after decalcification especially in samples with lower gelatin yield.

2. Materials and methods

2.1. Fish bone samples

Archaeological fish bones from the Nakatani location of the Hamanaka 2 site, Rebun Island, Hokkaido, Japan were used in this study. Hamanaka 2 site is a large multi-component shell-midden (Sakaguchi, 2007), and has been excavated several times in different locations since 1967 (Kato, 2015). Archaeological excavation of the Nakatani location was led by the Baikal-Hokkaido Archaeology Project (BHAP) and Core-to-Core Project since 2010, and yielded abundant archaeological remains (Kato, 2015; Müller et al., 2016; Weber et al., 2013). Fish bones used in this study were obtained from the Okhotsk (Layer III, 430–960 cal AD: Leipe et al., 2017) and Epi-Jomon (Layers VII and VIII, 2700–1500 cal BP: Weber et al., 2013) occupations. The Okhotsk layer is characterized as a well-stratified shell midden and contains human burials (Okamoto et al., 2016), skeletons of domesticated animals (dog and pig), ceramic and lithic (Lynch et al., 2018) materials, several plant remains (Leipe et al., 2017), and abundant marine mammal, fish, and shellfish remains. The Epi-Jomon layer is found within a densely-packed sand dune formation and contains dog skeletons, ceramic and lithic (Lynch et al., 2018) materials, remains of marine animals, and concentric hearth features. The cool temperature of Rebun Island (mean annual temperature in the period 1978–2002 was 6.6 °C)[1] and the presence of shell both promote good preservation of organic materials at the Hamanaka 2 site (Kato, 2015).

Fish bones were recovered by column sampling and subsequent water floatation with 9.52 and 4 mm mesh. After air drying, fish bones were stored in collection shelves and identified to genus, and, where possible, species by using reference fish collections in the Keio University, Japan. While numerous species were present, we focus on the most frequent fish taxa: vertebrae of cod (*Gadus* spp.), atka mackerel or hokke (*Pleurogrammus* spp.), and rockfish (*Sebastes* spp.), and maxilla/mandible of fugu (Tetraodontidae) were used in this study (Table S1). Although some cod bones could be identified as Pacific cod (*G. macrocephalus*), they are here subsumed into a generic *Gadus* category (Table S1). The total number of fish bone samples is 30 and that of analyzed subsamples is 68. One of the most frequent fish taxa, herring (*Clupea pallasii*), were not used in this study because their small bone size prevents replicate analysis.

2.2. Collagen extraction

In this study, effects of lipid extraction and order of NaOH treatment were evaluated in all samples and in the subset of cod bone samples, respectively. The first batch of fish bones from several taxa was processed with NaOH treatment before decalcification (method A). Taxa with low gelatin yield (< 3.5%: Ambrose, 1990) was used further for the evaluation of the order of NaOH treatment (see Table 1). This

[1] Original data were obtained via the web site of Japan Meteorological Agency. Measurements were made at the nearby Funadomari town in Rebun Island. http://www.data.jma.go.jp/obd/stats/etrn/view/annually_a.php?prec_no=11&block_no=1207&year=&month=&day=&view=p1 (accessed on 2018-04-13).

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