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An Enzymatic Method to Process Decomposed Non-Human Bone for Forensic DNA Analysis

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Abstract

Forensic analysis of DNA from non-human bones can be important in investigating a variety of forensic cases. However, decomposed bone is difficult to process for isolating DNA. In this study, a previously established enzymatic method was utilized to process bone samples that simulate decomposed specimens. Our results demonstrated that this enzymatic processing method is effective for removing decomposed soft tissues and outer surface materials such as mineralized bone connective tissue of bone fragment samples. Our data suggested that this method can be used in the initial sample preparation for cleaning the outer surface of decomposed non-human skeletal fragments. This study introduced an alternative method for processing decomposed non-human bone evidence prior to DNA isolation. Such a method can potentially be used to process various samples of different sizes and conditions for the investigation of a wide variety of criminal cases involving animals.

Keywords: Bone; Trypsin; Forensic DNA; Non-human

Introduction

The forensic DNA analysis of non-human bone DNA is a useful tool in investigating a variety of cases. Animal evidence associated to human victims or suspects and the killing, trade, and possession of an animal or animal products derived from a species that is protected from illegal hunting are two common applications of forensic investigations. The evidence is often examined using forensic DNA analysis to determine the species of the animal evidence. However, the success of DNA analysis of animal remains depends on the quality of extracted DNA. An animal killed illegally is often found partially consumed or decomposed in the field. Remains with postmortem decomposition pose a great challenge to forensic DNA analysis. The DNA extracted from the decomposed soft tissues is often degraded, rendering it unsuitable for species identification. Hard tissues such as bones are the preferred source for forensic DNA identification because the DNA of hard tissues can be protected from degradation. Thus, the forensic analysis of DNA from bone is important in species identification of non-human bone evidence.

It is required that the processing of non-human bone evidence follow the same standards as undertaken for any other forensic investigation [1]. One of the major problems is the removal of co-mingled remains, contamination by animal scavenging, environment-borne inhibitors, and bacterial contamination which interfere with forensic DNA analysis. As a result, the outer surface of the bone fragment must be removed [2]. Currently, limited methodologies are available for processing decomposed samples used in the forensic DNA analysis of non-human bone evidence. Most skeletal preparation techniques may cause DNA degradation, which is not appropriate for processing evidence intended for DNA analysis [3]. The processing of bones may be carried out by using a mechanical method [4]. However, to avoid cross-contamination between samples, the bone dust generated by the mechanical method (with single-use sanding discs attached to a rotary sanding tool during bone sanding) must be cleaned and removed. Thus, processing bone evidence obtained from a severely decomposed animal is sometimes a laborious and a time-consuming task [5]. Developing a simple and reliable processing method for processing decomposed evidence is highly desired.

An enzymatic method, using a proteolytic trypsin enzyme to degrade various types of proteins [6,7], has been utilized in the maceration of bone samples in skeletal preparation [8,9]. In our previous study, the trypsin maceration technique was adapted to prepare samples prior to DNA isolation from human fresh bone samples [10,11] and human burial bone samples [12]. Additionally, the effects of this technique on the yield of DNA isolated were compared to that of a mechanical method [12]. Comparable values of DNA yields between the two methods were observed [12]. This study adapted the enzymatic trypsin method to process decomposed non-human bones prior to DNA isolation. Swine (Sus scrofa domesticus) bones were used in this study since they are a useful model system for simulating various animal bones. Additionally, the bone samples studied were prepared to reflect more typically encountered samples in actual forensic cases. In this study, the effects of trypsin treatment on the yield of DNA isolated and on the quality of DNA analysis were examined.

Materials and Methods

Sample preparation and processing

The fragments of swine femur and scapula (approximately 250 g) were dissected. Experiments were prepared (Figure 1) by placing a piece of bone fragment with soft tissue, protected by a metal cage, outdoors for seven days (average daily high temperature, 32°C; humidity, 49%).

The surface cleaning of bone samples were processed using the trypsin method as previously described [11]. Trypsin (laboratory grade

powder) was obtained from Fisher Scientific. The trypsin treatment was carried out by placing a piece of bone fragment in 500 ml of trypsin solution (30 µg/µl, 10 mM Tris, pH 7.5) and then was incubated with gentle agitation at 55°C overnight. After incubation, the liquid was removed. To prepare untreated bone fragment as a control, the soft tissue of the bone was removed using a surgical scalpel. The bone fragments were further processed by inversion for 30 s in distilled water, 0.5% sodium hypochlorite, and 96% ethanol as described in Davoren et al. [4]. The bone fragments were then air dried.

For scanning electron microscopy (SEM) observation, samples were cut, dehydrated, and coated with gold under a vacuum according to the standard procedures. The samples were observed and photographed using a variable pressure scanning electron microscope (Vega 5136 mm) to confirm the cleaning effects.

Figure 1: Sample preparation of swine bones investigated in this study. Experiments were prepared by placing a piece of bone fragment (a fragment of swine scapula is shown), protected by a metal cage, outdoors for seven days.

DNA extraction and quantitation

Bone powder was prepared by drilling, as described in Courts and Madea [13], using a rotary tool (Dremel, Racine, WI). Demineralization of bone powder was carried out as described in Loreille et al. [14]. For each sample, 0.2 g of bone powder was decalcified by incubating in 3.2 ml of extraction buffer (0.5 M EDTA, 1% laurylsarcosinate) and 200 µl of 20 mg/ml proteinase K overnight at 56◦C with gentle agitation.

The DNA from each sample was extracted using the method previously described [11]. The volume of the demineralized sample was reduced to approximately 400 µl using an Amicon Ultra-4 (30 kD) column (Millipore, Billerica, MA). DNA was extracted using the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The final volume of eluted DNA was 60 µl. Extraction negative controls were employed to monitor potential contamination. DNA quantitation was performed using a NanoDrop 2000c spectrophotometer (Thermo Scientific,Wilmington, DE) according to the manufacturer's protocols.

Species identification by sequencing swine mitochondrial cytochrome b locus

The amplification of specific fragments of the swine mitochondrial cytochrome b (Cytb) gene was carried out. A 0.5 ng of DNA template was used. PCR reactions were performed in reaction volumes of 25 µl containing GeneAmp PCR Gold buffer (Applied Biosystems, Foster City, CA), 1.5 mM $MgCl_2$, 200 µM each dNTP, 1 mM bovine serum albumin (Sigma-Aldrich) and 2 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems). Additionally, 0.4 µM of each forward (5'-TCA CAC GAT TCT TCG CCT TCC ACT-3') and reverse primer (5'-TGA TGA ACG GGT GTT CTA CGG GTT-3') that amplify the Cyt b gene in vertebrates was used [15]. The expected size of the amplicon was a 521 bp fragment of the swine mitochondrial Cytb gene (at nucleotide position 524 – 1022; GenBank Accession Number: AY237533). The reactions were initiated with an 11 min activation step at 95°C. For each cycle, the cycling parameters included a 30 s denaturation step at 94°C, a 30 s primer annealing step at 50°C, and a 30 s extension step at 72°C. The PCR was performed for a total of 34 cycles. As a positive control, amplification with 0.5 ng of genomic DNA of known swine mitochondrial DNA sequence was carried out. To monitor contamination, PCR negative controls were included with each amplification experiment.

To identify and to quantify the PCR products, DNA separations were performed using the DNA 1000 Lab-on-a-Chip Assay kit with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) following the manufacture's protocol. The Agilent DNA 1000 ladder (Agilent Technologies) was used as a sizing standard. The data were analyzed to determine DNA fragment size based on the sizing ladder and internal standards. The quantitation of each PCR product was performed using the manufacturer's software provided with the Agilent Bioanalyzer 2100 system.

The 521 bp amplicon fragment of the swine mitochondrial Cytb gene was sequenced. The ExoSap-IT reagent (Affymetrix, Santa Clara, CA) was used to remove unincorporated primers and nucleotides. The cycle sequencing reaction was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The total reaction volume was of 20 µl including 5 ng of template. The reactions were initiated with a 60 s soak at 96°C. For each cycle, the cycling parameters included a 15 s denaturation step at 96°C, a 15 s primer annealing step at 50°C, and a 60 s extension step at 60°C. The cycle sequencing was performed for a total of 25 cycles. Post-amplification sample clean-up was carried out using the DyeEx spin columns (Qiagen, Valencia, CA). The cycle sequencing products were separated on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and were analyzed with the Sequencer software (Gene Codes, Ann Arbor, MI). The DNA sequence obtained was compared with the *BLAST* database.

Results and Discussions

The trypsin-treated bone fragments were examined after incubation. The removal of the decomposed soft tissue of the bone sample was observed after incubation (Figure 2). The surface cleaning effect of the trypsin treatment was further examined using SEM observation. Figure 3 shows the intact outer surface of untreated bone surface. The removal of the outer surface layer of the bone sample was observed after the trypsin treatment.

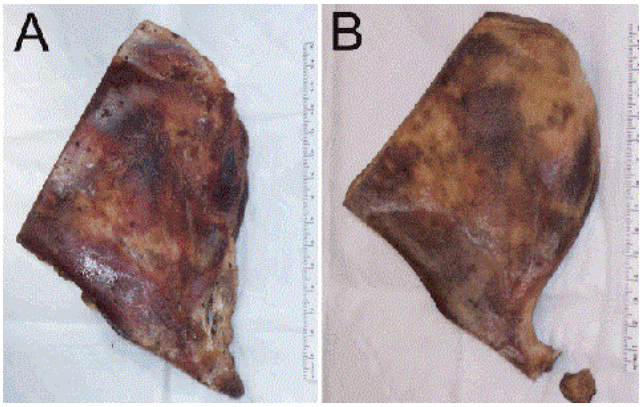

Figure 2: Enzymatic treatment of bone fragments. The enzymatic treatment was carried out by placing a piece of decomposed bone fragment in 500 ml of trypsin solution (30 µg/µl). The sample was then incubated overnight at 55°C. The trypsin-treated bone fragment was examined and photographed: A) Before, and B) after the trypsin treatment.

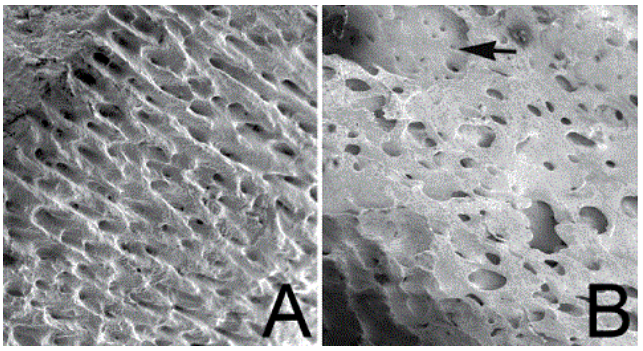

Figure 3: Scanning electron micrographs of control and trypsin-treated samples. Swine bone chips (outer surface of cortical bones; 0.2 g) were collected and examined using SEM: A) Untreated control sample (see Materials and Methods). The control sample showed the outer surface of intact plexiform bone tissue, and B) the trypsin (30 µg/µl) treated sample showed that the exposure of the vascular spaces of plexus (arrow), due to the removal of the surface layer of the bone sample, was observed. Field width: 18 mm.

DNA was isolated from trypsin treated samples according to the procedure as described in the Materials and Methods. DNA quantitation was performed, and the DNA yield of trypsin-treated bone samples was 1.68 mg DNA/g bone (the values were the means of six determinations), which was sufficient for subsequent DNA analysis. No DNA contamination was detected in negative controls. To evaluate the quality of DNA isolated from the trypsin-processed bone samples, species identification using mitochondrial DNA analysis was performed. In species identification, the loci most commonly used are the mitochondrial Cytb, cytochrome c oxydase I (COI), and D-loop loci [1]. In this study, a segment (521 bp) of Cytb gene was analyzed since it was applied to the identification of various vertebrates [16,17]. The Cytb fragment was amplified and quantified using a microfluid electrophoresis device: Agilent Bioanalyzer 2100. Successful amplification (average yield was 780 ng) was detected in all DNA samples tested. No adverse effect of trypsin treatment on PCR was observed compared to control samples (Figure 4). A cycle sequencing reaction usually requires approximately 5 ng of amplified product [18]. Thus, all amplified samples yielded sufficient quantities of PCR products for subsequent sequencing analysis. The amplified fragment at the Cytb locus was successfully sequenced (Figure 5). No adverse effect of trypsin treatment on sequencing was observed compared to control samples (Figure 5). Results from the sequence analysis confirmed that the origin of the samples was Sus scrofa domesticus (465 bp, E-value = 0.0).

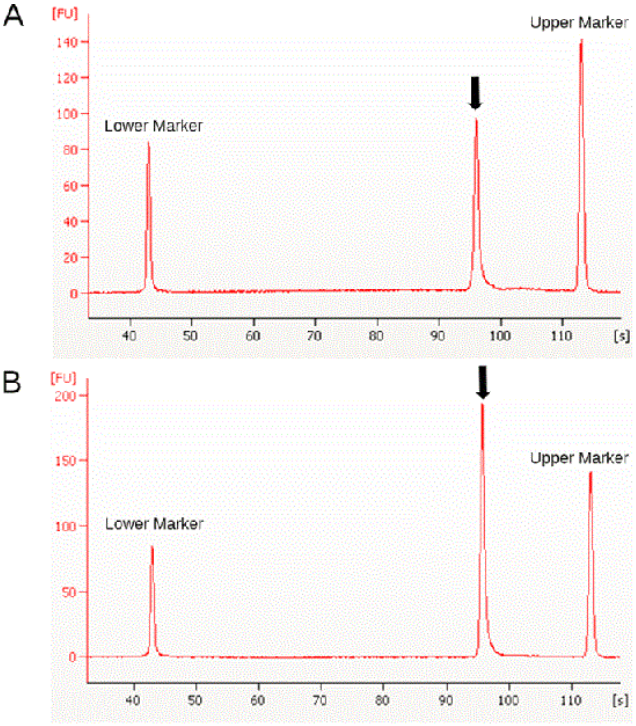

Figure 4: Results from the Agilent Bioanalyzer 2100 showing electropherograms with the mtDNA Cytb amplicons (arrows). The x-axis on the electropherogram represents the migration time of the amplicon and the y-axis represents the fluorescence intensity of the amplicon. RU: relative fluorescence unit. S: second. Lower marker (15 bp) and upper marker (1500 bp) are the internal size standards. A) Untreated control sample (see Materials and Methods) and B) the trypsin (30 µg/µl) treated sample.

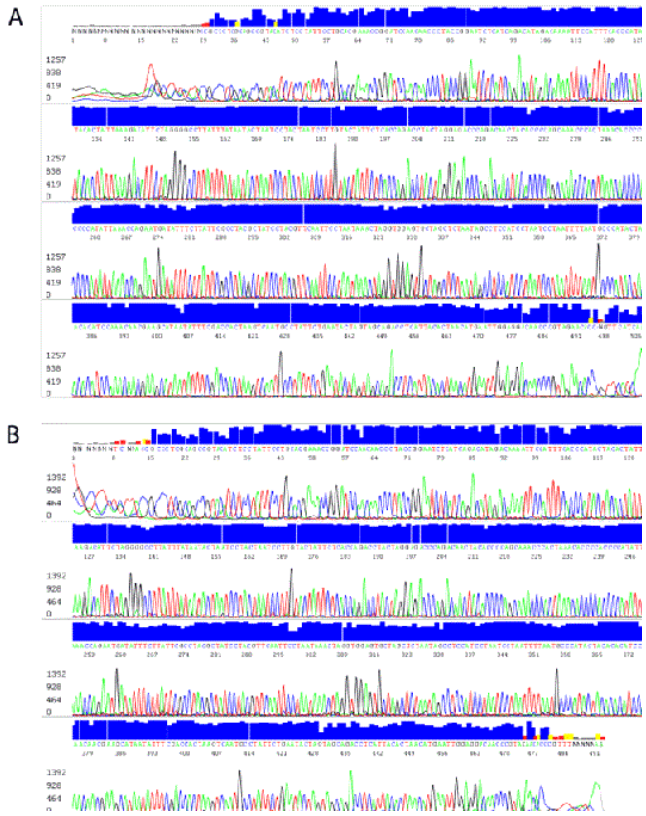

Figure 5: Confirmatory DNA analysis using direct sequencing of amplified fragment at Cytb locus. The electropherograms of A) the untreated control sample (see Materials and Methods) and B) the trypsin (30 µg/µl) treated sample.

Currently, the cleaning of the outer surface of bone fragments (removing approximately 1–2 mm of surface bone materials) for forensic DNA analysis is usually carried out using mechanical methods, such as sanding using sanding discs attached to a rotary tool [4,13,19] or sandpaper [20,21]. However, mechanical methods cannot be used to process multiple samples simultaneously. Additionally, mechanical methods cannot be used to process bone samples that are porous or fragile [18].

In a previous study, an enzymatic method using trypsin solution was adapted to clean bone samples prior to DNA isolation from fresh swine and human bone samples [10]. It was demonstrated that this trypsin method can remove outer surface materials such as the mineralized bone connective tissue of fresh bone samples [10]. A separate study revealed that the yield of DNA isolated from trypsin-treated fresh human bone samples was sufficient for forensic short tandem repeat (STR) analysis [11]. In a subsequent study, the trypsin method was studied in samples that are more typically encountered in actual forensic cases such as human burial bones (over 50 years post-mortem). Comparable values of DNA yields and Internal Positive Controls (monitoring the presence of PCR inhibitors) between the mechanical sanding and enzymatic trypsin method were observed. Additionally, the effects of the trypsin method on the quality of STR profiling were also studied. The percentage of the allele calls of STR profiles and the signal intensities of STR alleles were comparable between the two methods [12].

In this study, the feasibility of using the enzymatic trypsin method for cleaning decomposed bones prior to DNA isolation was examined. Our results demonstrated that this method was effective for removing decomposed soft tissues attached to bone samples and the outer surface materials such as the mineralized bone connective tissue of bone fragment samples. Our data suggested that this method can be used in the initial sample preparation for cleaning the outer surface of decomposed non-human skeletal fragments. This study introduced a new method for processing decomposed non-human bone evidence prior to DNA isolation. Our method can be advantageous over conventional methods. First, this method is not labor-intensive for processing bone samples. Second, this potentially automatable method can be used to process multiple samples simultaneously to improve the throughput. Additionally, such a method can potentially be used to process various samples of different sizes and conditions (i.e. porous surface or fragile) for the investigation of a wide variety of criminal cases involving animals.

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