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The Use of Trypsin to Prepare Skeletal Material for Comparative Collections with a Focus on Fish

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ABSTRACT: There are skeletal collections in museums and universities throughout the world that are not useful to the zooarchaeologist because they have not been properly cleaned–often the result of having been processed in the field. In this paper, a method to clean the bones of smaller animals is presented that is both quick and efficient with a minimum of special requirements. This method was designed to work under field conditions to create reference collections of local fauna. Three objectives were considered: a) preparing high quality skeletal material, b) minimizing the amount of specialized equipment needed to do so, and c) having a process easy to perform under non-laboratory conditions. The method developed involves maceration by the use of the enzyme proteinase trypsin. This form of maceration is easy to use and has given excellent results both in the laboratory and in the field. It has been used to prepare skeletons of small mammals, birds, reptiles, amphibians, and especially fish.

KEYWORDS: ZOOARCHAEOLOGY, COMPARATIVE COLLECTIONS, MACERATION, ENZYMES, TRYPSIN, SKELETAL PREPARATION

RESUMEN: Existen colecciones esqueléticas en museos y universidades por todo el mundo que no resultan de utilidad al zooarqueólogo debido a que no han sido adecuadamente preparadas. Ello es resultado de que los restos hayan sido frecuentemente procesados sobre el terreno. En este trabajo presentamos un método para preparar los huesos de los animales de menor tamaño que es rápido y eficiente y con un mínimo de requisitos específicos. El método fue diseñado para crear sobre el terreno colecciones de referencia de la fauna local. Tres objetivos fueron considerados: a) preparación de un material esquelético de alta calidad, b) minimizar los requisitos de instrumental especializado requerido para llevar esto a cabo y c) disponer de un procedimiento fácil fuera del laboratorio. El método nuevo desarrollado implica la maceración con el uso de la enzima tripsina. Este tipo de maceración es de muy fácil uso y ha proporcionado excelentes resultados tanto en el laboratorio como en el campo. Se ha utilizado para preparar esqueletos de micromamíferos, aves, reptiles, anfibios y muy especialmente peces.

PALABRAS CLAVE: ZOOARQUEOLOGÍA, COLECCIONES COMPARATIVAS, MACERACIÓN, ENZIMAS, TRIPSINA, PREPARACIÓN ESQUELÉTICA

INTRODUCTION

The aim of this paper is to describe in detail a process for removal of soft tissue from skeletal material that is suitable for preparing specimens for comparative osteological collections under both laboratory and field conditions. The technique, which involves the use of the protease enzyme trypsin, is relatively simple to employ and minimizes the need for specialized equipment, thus making it particularly suitable for use in the field. The resulting skeletal elements maintain their structure without being brittle and are suitable for long-term storage. Trypsin is found naturally in many animals as a digestive enzyme for breaking down protein and, together with other protease preparations, is widely employed commercially. Among its uses in the laboratory have been the preparation of cleared and stained specimens (Taylor, 1967) and, in a limited way, the cleaning of small vertebrate skeletons (von Endt *et al*., 1999).

PREPARATION METHODS

There are numerous methods of skeletal preparation that have been used through the years. These range from burying specimens in the ground or in compost heaps, to processing through water maceration and with detergents, to using specific enzymes in solution. The use of chemicals to clean and degrease bones has been widespread but has not always been successful; many of the chemicals have since been found to be carcinogenic, and some of the combinations are plainly dangerous (Anderson, 1965; de Wet *et al.,* 1990). Commercial pre-soak laundry detergents containing various enzymes have been promoted (e.g., Ossian, 1970; Groen, 1984), but one of these, BIZ, has been used repeatedly without a proper understanding of enzymes and how they function. It is necessary to stop the action of the enzyme or denature the enzyme after digestion has been completed. If this is not done, the enzyme can continue to digest the bone protein and amino acids, which will eventually destroy the specimens. Denaturing can be accomplished using heat, ethanol, and strong detergents. Because of the need for constant monitoring of individual skeletal parts, the use of BIZ and other like products is not recommended.

More satisfactory are the following methods (e.g., Hill, 1975; Hefti *et al*., 1980; Davis & Payne, 1992; Sullivan & Romney, 1999):

a) Cold Water Maceration

Fleshed-out body parts are put into a container large enough to be covered with water where they remain until the flesh has fallen away from the bone. This takes from a few days to several weeks and is highly odoriferous.

b) Hot Water Maceration

This process involves putting bones in containers that can be heated and then «simmered», but not boiled. The application of heat greatly speeds up the process when compared to cold water maceration, but must be attended to so damage to the bones does not occur. Completion of the process can take from a few minutes to several hours.

c) Beetle Farms

This method involves defleshing and drying the bones and then placing them in a container with a screen cover. The container holds a colony of dermestid beetles that will feed on the remaining flesh and clean the bones. This method can take many hours to several weeks and is particularly suitable for larger specimens. Left unchecked, dermestids will consume cartilaginous skeletal parts and even less dense portions of bones. It is thus not recommended for preparing the skeletons of small animals unless the process is carefully monitored.

d) Maceration using protein digestive enzymes

There are a number of enzymes that have been used for this purpose. Those discussed here are Neutrase (briefly) and Trypsin (at length), both used in the food-processing industry. Simonsen *et al*. (2011) have evaluated four other enzymes by experimentally macerating mice and rat skeletons.

Neutrase Maceration

Davis & Payne (1992) and Baker *et al*. (2002) recommend using the enzyme Neutrase. This enzyme comes in liquid form, is made into a water solution, and is most effectively used at about 45º–50º C (but works, albeit considerably less effectively, at as low as 30º C). Defleshed bones

are submerged in the solution until the remaining flesh is digested. This process takes between 6 and 12 hours, with 2 to 3 solution changes, at about 45º C to produce clean specimens, with longer times and more solution changes needed for larger and older animals.

Neutrase is somewhat cumbersome to use in the field because it comes in liquid form, needs refrigeration to remain stable, and ideally requires a temperature level for the working solution that is beyond the limits of simple heating devices (e.g., an aquarium heating element that is usually limited to about 38º C [100º F]). Experiments carried out by the first author have confirmed that Neutrase works well when optimum conditions are met. However, in the absence of refrigeration and of the ability to reliably and safely heat solution containers to ca. 45º C and maintain that temperature for many hours, dependence on Neutrase for skeletal preparation may be problematical especially under field conditions.

Trypsin Maceration

Another enzyme that has been used occasionally in the past to deflesh skeletons is Trypsin (von Endt *et al*., 1999). The remainder of this paper deals with the first author's experience employing aqueous solutions of this enzyme. The benefits of its use are that Trypsin is supplied in dry form and thus is easy to transport, is stable at room temperature, works very well at about 37º C (ca. 98º F), and produces excellent, stable results.

MATERIALS AND METHODS

Materials

Water (H_2O) – needs to be reasonably clean (distilled water is preferred but not pH test strips). The proteolytic enzyme Trypsin (e.g., Fisher Scientific T-360) that comes in powder form Alconox™ (a glassware detergent). Waterproof paper (for making labels) and a pencil. Fine-tipped tweezers. A fine strainer/screen/sieve (1 mm mesh size).

Several glass jars with rubber seals in assorted sizes, both 1000 ml (1 l, e.g., Le Pratique canning jars) and smaller sized jars with airtight lids for smaller animal parts.

Latex or other waterproof protective gloves.

Beakers and columns for mixing chemicals:

1 – 500 ml graduated column

2 – 2 l polypropylene measuring pitchers

2 – 1.9 l measuring cups.

A lab dissection kit, and/or fish or carcass cutting knives.

A microwave oven (in the lab) or a steamer (in the field).

Heating equipment for use in the lab:

1 – 10 gallon or 40 l storage tub with a cover for the water bath (e.g., RubberMaid™)

Aquarium heater–38º C (100º F)

Aquarium circulator pump.

Heating in the field:

Warm ambient air temperature or exposure to the sun

Trypsin Enzyme Solution:

Basic proportions for 1 liter (1000 ml) of solution

Powder = 12 g (by weight) Trypsin

Liquid = 700 ml H_2O (water) to start; top off to 1 liter once the Trypsin is dissolved.

General Method

Using a column, measure out the water into a container that is suitable for pouring. Add the Trypsin to the liquid. Trypsin takes some effort to dissolve in the water, which can be facilitated by pouring the mixture back and forth between containers until the powder is dissolved. Once the Trypsin is in solution, fill the jars that contain the bones and their waterproof labels, leaving a little space for expansion at the top. The specimens are now ready to process. To be a catalyst, enzymes need the proper temperature, which in the case of the Trypsin solution seems to be optimum at ca. 37º C (98º F), although the solution will work at temperatures as low as 30º C (80º F). Maintaining optimum temperature can be effected by different methods, and the preparator can use whatever

method best suits the situation. For example, there are heating pads used for germinating seeds on which the jars can be set. In the field, the sun can be used as a heat source if the ambient temperature is not too low. Finally, there is the combination of a large plastic container (e.g., Rubbermaid) filled with water, an aquarium heater with temperature adjustment capability, and an aquarium pump that circulates the water. The first author has found that the water bath method works well, with the size of the container permitting one to process several jars at a time.

Specific Method with a Focus on Fish

The following sequence is described for fish, but can be used for most any small animal. It should be noted that, throughout processing, the preparator must remember to keep different parts of the body separate and with an appropriate waterproof label.

Step 1: Initial preparation

Each specimen is prepared individually. It should already have information recorded about provenience, date of capture, place of capture, taxon, etc. and may have been given a specimen number. If it does not have a specimen number, then one should be assigned before the preparation process begins, either in the form of the final collection number or a temporary lab or prep number. Fish and other animals will usually be frozen when beginning the preparation process. The specimen is thawed, measured, and weighed using an electronic or other scale. Such information, together with any other relevant data, is entered into a lab notebook next to the assigned number. This number is then written in pencil on pieces of waterproof paper one of which is included with each part of the specimen that is to be processed separately (see below). If the fish has scales, remove them to the degree possible and place them in a small container with a prep tag. Next, an incision is made in the stomach area of the fish making sure to penetrate the stomach itself. The specimen is rinsed, left wet, and placed in a 4 ml plastic zip bag along with the prep number. If using a microwave to heat the specimen, do not seal the bag completely and use a microwave-safe plate. There is no specific amount of time specified for heating a specimen in a microwave oven. Approach this procedure in a conservative manner with common sense. For very small fish, first try 30 seconds; for larger specimens, additional minutes may be necessary. Specimens can also be heated in a stovetop steamer, in an oven over a water-filled tray, or in a saucepan partly filled with water. The purpose of this «light cooking» is to solidify the tissue to make it easier to remove from the bones, as well as to melt fats and release oils before using the enzyme solution.

Step 2: Separating the parts

Separate the head and tail from the body. Remove as much flesh as possible, feeling for bones as you go. Take the head apart and put all the head bones in one jar with a label. Put the tail in another jar, again with a label. Remove the fins, prepare labels for them by name and side (right or left), and put them in separate jars. Remove the skin and scales and put them into a jar with a label. Put ribs and all other bones in as many jars as needed, again removing as much flesh as possible before doing so and including a label in each jar. Avoid crowding materials within the prep jars; otherwise, maceration may not be successful. The minimum ratio of body material to solution is 1:1, but it is best as a ratio of 2:1 liquid. This is especially true for oilier species of fish or other animals.

Step 3: Using the solution

Mix the enzyme, using 12 g of Trypsin to make 1000 ml of solution. Use distilled water if available with a pH between 6.5 and 7.0. If macerating in the field, use clean water and check its pH. Mix until the Trypsin has completely dissolved in the liquid. This is done most easily by pouring back and forth between two containers. Top off the mixture with water to 1000 ml. Fill the jars containing the fish parts, leaving some room at the top of each jar for expansion. During enzyme maceration, keep the solution at a temperature of about 37º C (98º F) using one of the techniques described above. The process will normally take 6–9 hours. If the digestion has not been completed within that

time frame, drain the relevant jars through a 1 mm sieve, add more solution, and repeat the process.

Step 4: Completing the process

When the bones are free of flesh and cartilage, or digestion is completed to the degree desired, pour the contents of the jar into a 1 mm sieve and rinse gently with warm water at 30º C (ca. 86º F). Use tweezers to pick bone out of the residue. Rinse the jar, add a rounded tablespoon of the lab glassware cleaner Alconox (for a 1000 ml jar), put the bones back into the jar, add warm water, seal, and agitate gently to dissolve the Alconox soap powder. Use a rounded teaspoon for jars smaller than a liter. Initially, the Alconox solution becomes cloudy due to lipids and soap particles; when it clears, degreasing is complete, which may take from 1 to 12 hours. At that point, pour the jar contents into a sieve and thoroughly rinse with warm water at 30º C (86º F), spread out the bones from each jar on separate plastic trays covered by paper towels, and dry slowly, for at least 24 hours, at room temperature. Remember to keep the labels that were in the jars associated with their original specimens. Note that the use of Alconox is a critical step in enzyme maceration, because it serves both to neutralize the Trypsin and to dissolve the lipids.

Specific Method: The Use of an Enzymatic Gel

List of ingredients:

Methocel A (Dow Chemical) (2g)

Warm water (30 ml at 30^0 C [86^0 F])

Trypsin solution (500 ml).

Because the structure of fish fins is delicate, an enzyme solution that has a very liquid composition can dissolve the tissue that holds the rays together. This may be an issue should it be desirable to maintain the overall structure of the fin. To better control enzyme activity, localized surface application of an enzymatic gel is an effective way to reduce enzyme concentrations on the surface of the fin. This is effected by Trypsin being suspended in a neutral material, such as the cellulose ether product Methocel A (Dow Chemical), before being applied to the area to be cleaned. This methylcellulose is a carbohydrate polymer that will dissolve in cold water and in certain organic solvents by swelling and eventual hydration. The viscosity of the gel can be adjusted by changing the ratio of Methocel to enzyme solution.

To make the gel, make 500 ml of the Trypsin solution, using 6 g of Trypsin in 500 ml of warm water. Add 2 g of Methocel A to 30 ml of warm water and mix until the particles are thoroughly wetted and a smooth paste is obtained. Add 70 ml of the Trypsin solution to the paste and mix until a smooth gel results, which may take a few minutes.

This enzyme gel is used as a protease «stripease» and can be made in any strength or viscosity desired. If a more viscous gel is wanted, use 3 g or 4 g instead of the 2 g of Methocel A noted above. Once the gel has been made, it can be maintained in a refrigerator for a couple of days. Application to the fish fin requires the gel be warmed to 37^0 C (98º F) to ensure that the enzyme is at its optimum working temperature.

It is a good idea to keep the gel warm during its period of application. This can be done with a lamp or heating element. The area should be covered with plastic wrap to prevent evaporation of the gel and the liquid within it. Once the desired degree of maceration has occurred, gently rinse the fin with cold water to remove the gel or gently peal the gel off the surface and then gently rinse to remove any residue.

The use of an enzymatic gel is particularly helpful for cleaning areas of articulation that contain cartilage and small bones. Although care should be taken in its application and the progress of enzyme activity closely monitored, the use of a gel does provide the opportunity to better localize and control enzyme activity and thus to preserve skeletal articulations when such are desired.

Special Case: Rehydrating a Specimen

Fish and other specimens in many natural history museums have been preserved in alcohol. To skeletonize these requires that they be rehydrated before using an enzyme maceration procedure. The first author had the opportunity to test such a procedure on a common carp (*Cyprinus carpio*) from Harvard's Museum of Comparative Zoology (MCZ#2120), which had been stored in alcohol

since the early 1800s. Specimens stored in this manner can be rehydrated and macerated if they have NOT been treated with formalin−a solution of formaldehyde (HCHO), 37% water and 10-15% methanol (CH_3OH)−that, when used to preserve organic specimens, will prevent maceration. The rehydration process is to replace with water the alcohol that has saturated and stiffened the specimen. Rehydration requires a large container filled with water at room temperature; the larger the volume of water the better. Immerse the specimen and change the water twice daily for five days or until the smell of alcohol is gone. Even after this length of time, some of the deepest tissues may still contain alcohol. The only way to overcome this is to expose as much of the tissue as possible to water, which may involve cutting open the specimen in a strategic fashion. When rehydration seems to be complete and the specimen is ready to be «lightly cooked» in a microwave, proceed slowly at first, a minute or two at a time. If the scales curl, which indicates that they are not completely rehydrated, carefully remove them, put them in a jar of distilled water, and let them soak for several hours. Start to deflesh as much as possible even though the flesh may seem dry and stringy. If the flesh is too difficult to remove or if there is an odor of alcohol, put the specimen in water, distilled if possible, and soak overnight. This resoaking process may have to be repeated several times before defleshing can be completed. In any case, it is a good idea to soak the defleshed parts of the skeleton overnight before employing enzyme maceration, as previously described above.

Processing Reptiles, Amphibians, and Birds

When processing smaller animals such as reptiles, amphibians, and birds, using the Trypsin enzyme method discussed above, some special procedures should be considered. Particularly important to consider is that the bones of the feet are particularly small and thus must be handled with great care. Some of the difficulties encountered and possible solutions are discussed below based on the preparation of a Nile monitor lizard (*Varanus niloticus*), diamondback terrapin (*Malaclemmys terrapin*), and sharp-shinned hawk (*Accipiter striatus*),

Lizards and Snakes

Removing the skin from reptiles is much easier when they are fresh and more difficult after they have been frozen. After thawing, if necessary, cut around the lower part of each limb and leave the skin on the front and rear feet like gloves. Since the enzyme will not dissolve the skin of reptiles, leaving the skin of the feet intact keeps the foot bones together. Remove the internal organs and rinse, leaving the body wet. Make sure the specimen has a preparation or other identifying number, make a tag, and put these together into a 4 ml plastic zip bag for «light cooking» in a microwave. It is best to microwave in a series of steps, starting with 10 minutes (in the case of the monitor lizard, a shorter period for a smaller animal), checking whether the meat can be removed readily from the bone, and then proceeding in short intervals until the specimen seems ready. Reptiles are very much like birds in that, when cooked, the texture of the muscle tissue is very similar. Do not overdo the cooking procedure and test frequently. Remove the meat as one would from a chicken, being careful not to break any of the small bones or disarticulate the skeleton other than to separate the specimen into the four legs, four feet, head, ribs, and vertebral column. Put each part into a separate jar with a cover along with its own descriptive waterproof tag (e.g., R-12, leg, left front). To keep the vertebrae in the proper order, a thin wire can be inserted through the neural canal; this is particularly important in the case of snakes. The parts that are cartilage, as at epiphyses, will be dissolved by the enzyme process. Fill the jars with enzyme solution, and place them in the water bath. It may take some time for the tissue to disappear completely. If they are not clean after 12 hours, pick the bones as clean as possible before putting them in a new batch of enzyme solution. At this point, check the feet from which the skin was not removed. If the phalanges and metacarpals are not clean, cut the skin down each toe. This will let the enzyme reach the terminal phalanx. Once the bones are clean, the skin can be removed. When pouring off the solution, use a very fine-screen sieve such as a tea strainer to catch the smallest bones of the feet. The easiest way to rinse the skeletal portions is to leave them in their jars, drain carefully, fill with warm water, and repeat the process until they are clean. The next step is to degrease and neutralize using Alconox, rinse, and dry as described previously. This same procedure can be used for frogs and snakes.

Turtles

There are two approaches to exposing the interior parts of turtles. One method is to heat them in a microwave oven before trying to separate the plastron from the carapace. The other method involves separating the two parts by cutting the connections between them. The major connection is called the bridge, which in some cases is «soft» and can be cut with a sharp knife; otherwise a handsaw or a DREMEL tool may be used. In either case, the skin that forms the seal around the neck, legs, and tail must be cut free of the carapace and plastron. After the interior parts have been exposed, put the turtle in a 4 ml plastic bag, heat it in a microwave, and remove the tissue from the bones. Internally, while there are not so many bones as in other vertebrates, you still may want to remove the bones in individual groups and label them separately. Process the legs singly as was done for the lizard. Find a large plastic food container with a cover within which the carapace can fit and put the plastron in first, then the carapace. If it is necessary to save space, put the skull, cervical vertebrae, and tail, or caudal vertebrae, inside the carapace; otherwise, process them separately. Mix the enzyme solution as above and fill the container, making sure to cover the carapace. The skin of turtles is like that of lizards in that it does not dissolve in the enzyme. It is thus important to remove as much skin as possible from the head. Also, there is a keratin covering on the carapace that will not dissolve but can be peeled off. Rinse and follow the degreasing procedures previously described and let dry.

Birds

Birds are prepared in basically the same manner as lizards, the difference being the need to remove the skin and feathers. Feathers are also made of keratin and will not dissolve in an enzyme solution. When skinning, be especially careful of the bones in the wings and legs of smaller specimens. Again, it is helpful to keep separate and individually label the right and left wings and right and left legs. There are some cartilaginous parts of birds that will dissolve, although this not a problem for most zooarchaeological purposes, since in most cases those parts will not be preserved in the archaeological record.

CONCLUSIONS

One of the principal purposes for developing the Trypsin-based maceration technique was to make it workable in the field. The late Stine Rossel, when a graduate student in archaeology at Harvard University, used the techniques described here during two field seasons at Abydos and Aswan, Egypt. She reported (personal communication, 2004) soaking fish carcasses in hot water, at about 95º C (203º F), for 5 to 20 minutes to make the flesh easy to remove. The Trypsin solution was prepared in the usual manner, and degreasing was done with Alconox. In her case, the ambient air temperature was so warm that no hot water bath was needed. The method proved easy for her to use and gave very acceptable results. Even though concentrations and other parameters could not be controlled as well as they would have been in a laboratory setting, the results appear to have been just as good. Because Trypsin and Alconox are dry chemicals, Rossel was able to safely carry sufficient quantities of both to Egypt to prepare a comprehensive comparative collection of Nile fish.

As noted above, there are many ways to skeletonize animal carcasses. Enzyme maceration is particularly suitable for smaller animals, being quite effective and relatively quick. Using Trypsin as the enzyme of choice takes advantage of it being supplied in dry as opposed to liquid form, being stable at room temperature and thus not requiring refrigeration, and having a relatively low and thus safe optimal working temperature of about 37º C (ca. 98º F). It produces high-quality specimens that remain stable over time so long as a degreasing agent such as Alconox is used at the end of the preparation process to neutralize the Trypsin and to dissolve the lipids that are a side product of enzyme maceration.

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