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TECHNICAL NOTE

ANTHROPOLOGY

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A Fast Preparation of Skeletal Materials Using Enzyme Maceration*

ABSTRACT: The current study investigates the removal of soft tissues from mice and rats by the use of three different proteases and one lipase from Novozymes A/S. The results demonstrate the enzyme maceration to be remarkably fast (1–3 h) compared to the traditional warm-water procedure, which requires up to several days. In addition, the enzyme maceration eliminates the odor problem associated with the traditional procedure. It is shown that stirring of the enzyme maceration bath is the main factor which determines the speed of the maceration. For mice, the time required for enzyme maceration can vary from 1 to 8 h depending on the stirring speed. The method investigated here allows preparation of skeletal material in an essentially odorless way within a matter of hours, making the method useful in particular for forensic science, private conservation workshops, and educational purposes.

KEYWORDS: forensic science, skeletal preparation, enzymes, maceration, fast method, bones

The cleaning of bones is a common practice in the fields of forensic science, conservation, zoology, anthropology, and in the preparation of hunting trophies (1–8). Museum curators make use of maceration techniques to remove soft tissues in connection with exhibitions and dry storage of skeletal materials, and both professionals and students in conservation and medical science have the need to prepare bones or skulls for educational and scientific studies (6,9–12).

Among several standard maceration techniques (7) (i.e., insect consumption, cold-water and warm-water maceration), one of the most commonly used methods is warm-water maceration, a technique that relies upon bacterial decomposition of the flesh and soft tissues (1,5,13–15). In this technique, the body or part of the body is skinned and eviscerated and subsequently placed in lukewarm water at 37–45°C. Warm-water maceration requires between 2 days and 8 weeks depending on the temperature and the amount of bacteria present in the animal (3,5). Bacteria-filled water from earlier macerations can be added to speed up the process. During the warm-water maceration, an obnoxious smell develops and facilities for ventilation are required.

Subsequent to maceration, some museums cook the bones in aqueous sodium carbonate solution to get rid of the smell and the greasy layer on the bones (7,16). However, such heat treatment may adversely affect the ability to retrieve DNA from the sample in future research (6,7,13,16,17).

Some studies have dealt with alternative maceration methods involving the use of commercial detergent (e.g., Persil, "Biz," Ariel) (4,7,9,11,16). Detergent maceration makes use of the enzymes present in the cleaning agent, and an increased speed of maceration and removal of bad smell have been observed. However, the exact composition of commercial detergents is often proprietary knowledge and not directly available. Besides various kinds of enzymes, the detergents also contain tensides, builders (inorganic complexing agents), additives, bleaching agents, and corrosion inhibitors (2,4,18–21). The aggressive mixture in detergents may cause damage to the specimens, and decalcification, softening, and transparency of detergent-macerated bones have thus been noted (18,19,22). Other maceration techniques make use of enzymes from various sources such as papain from papaya fruit or a mixture of digestive enzymes like pancreatin, which contains both amylopsin, pancreatic lipase, and the protease trypsin (4,7,11,23,24).

In the current study, we have investigated the use of commercial proteases and lipases from Novozymes A/S to perform enzyme maceration on mice and rats. The enzymes used in laundry detergent were received as aqueous solutions so harmful dust from pure enzyme powder was avoided, and the health and safety guidelines for working with enzymes could easily be followed. The experiments have shown that enzyme maceration using a dilute aqueous mixture of protease and lipase can be performed within hours.

The enzyme maceration technique is practically odorless which makes it particularly useful at educational institutions and small private conservation workshops. Enzyme maceration may also be useful in forensic science and in museums if time is an issue and when part of a skeleton or a small number of specimens are to be processed.

Materials and Methods

The mammals used were white NMRI laboratory mice (*Mus musculus*, gender: male) and white WISTAR laboratory rats (*Rattus*

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norvegicus, gender: male). The specimens were received in frozen condition. Each specimen was thawed overnight in a refrigerator at 5°C, then skinned and eviscerated, and its weight was recorded. The specimen was then refrozen and kept at −18°C until used. In all, 59 mice and eight rats were examined in this study.

The enzyme solutions from Novozymes A/S used in this research were lipase Lipex® 100 L and three proteases Savinase® 16 L: Type EX, Liquanase® 2.5 L, and Alcalase® 2.4 L FG (Novozymes A/S, Bagsvaerd, Denmark). The enzyme solutions were received in 1-L plastic containers and stored in a refrigerator at 5°C until used.

Most of the experiments were performed with Savinase® 16 L, Type EX. With this protease, 37 mice were macerated. The large number of experiments were carried out to repeat the experiments as double and triple determinations. In the series with the two other enzymes and mice, and the series with the rats, only single determinations were performed. This was performed to reduce the number of animals used because only tendencies and not statistical results were necessary.

Before maceration, the specimens were thawed in a beaker containing ordinary tap water for 1–2 h. The water was then discarded and warm tap water was added and the beaker plus content was placed on a magnetic hotplate stirrer and warmed to 55 ± 5°C. The enzyme solutions were then added (see later section for actual amounts), and the mixture was stirred at 55 ± 5°C until the maceration was finished.

To compare the effect of the different enzymes and the speed of maceration, it was necessary to define a point where the maceration of the specimen was finished. This was defined as the point in time, with resolution of 15 min intervals, where the skull and jaw are separated and tongue and cerebral matter were dissolved. In addition, the skeleton will at this stage be completely disarticulated, with all texture and tendons being dissolved.

When the maceration was finished, the maceration mixture was filtered through a coffee filter placed on a sieve to collect all the skeletal material. Ten milliliters of 36% hydrochloric acid was added to the filtrate to denaturate and destroy the enzymes before the solution was discarded.

The skeletal material was returned to the beaker again and thoroughly rinsed in running tap water for 15 min and finally rinsed with deionized water. The skeletal material was then placed in Petri dishes, covered with wet paper towel, and left for slow air-drying to prevent deformation of the bones.

Maceration of Mice

All experiments with mice were performed in 600-mL beakers and a total volume of the reaction medium water + protease + lipase = 300 mL.

Stirring

Because the maceration time may depend on stirring of the reaction bath, three experiments with varying speed of the magnetic stirring were performed. The reaction medium contained 260 mL of tap water and 20 mL protease (Savinase® 16 L: Type EX or Alcalase® 2.4 L FG) + 20 mL lipase (Lipex® 100 L). Owing to variation of the stirring speed between the different magnetic stirrers, the stirring speed was defined by the length of the vortex in the mixture before adding the specimen. Fast stirring gave a vortex reaching the bottom of the beaker and was the maximum stirring speed possible before the stirrer bar became uncontrollable. Slow stirring gave a vortex of ca. 2 cm from the top. The results are shown in Table 1.

TABLE 1—*Maceration time in hours as function of stirring speed for mice.*

	Savinase		Alcalase
Stirring Speed	Experiment 1	Experiment 2	Experiment 1
Fast	1	1	1
Slow	6	5	5
None	8	>7	>7

TABLE 2—*Maceration time in hours for mice as function of the amount of Lipase used.*

			Maceration Time/Hours	
Lipase/mL	Savinase/mL	H_2O/mL	Experiment 1	Experiment 2
10	5	285	1¾	1¾
5	5	290	1.5	1¾
2	5	293	1¾	1¾
10	10	280	1	–
5	10	285	1	–
2	10	288	1	–

The results in Table 1 clearly indicate that the stirring speed is a major factor in the maceration time. When stirred fast, the specimen spins around in the beaker and this motion helps loosen the soft tissues, tongue, and cerebral matter in particular. Fast stirring speed was therefore used in all remaining experiments.

Lipase

The amount of lipase enzyme needed in the maceration process was investigated by running two series where the amount of the protease enzyme (Savinase® 16 L: Type EX) was kept constant at 5 and 10 mL, respectively. The amount of lipase (Lipex® 100 L) was either 2, 5, or 10 mL and the amount of water varied to give a final volume of 300 mL in the reaction medium. The results are collected in Table 2.

The results indicate that the time required for maceration is independent of the amount of lipase and only depends on the amount of protease used. However, the filtration process was much easier when 10 mL of lipase was used compared to experiments using only 2 or 5 mL. When small amounts (2 or 5 mL) of lipase were used, the filtrate was greasy and clogged the filter repeatedly. This was avoided by using 10 mL of lipase where the solution ran through the filter without any problems.

Protease

To investigate the maceration time as function of the type and amount of protease used, a series of experiments with varying volumes of proteases was performed. In each experiment, the following parameters were used and kept constant: the volume of Lipex® 100 L was 10 mL, the total volume (lipase + protease + water) was 300 mL, the temperature was kept at 55 ± 5°C, and maximum stirring speed was used. Three proteases (Savinase® 16 L: Type EX, Liquanase® 2.5 L, and Alcalase® 2.4 L FG) were used in varying amounts from 0.1 to 9 mL. A control using only a solution containing 10 mL of lipase and 290 mL of water gave a maceration time of 6½ h. This value was used as zero value in all three series and the results are shown in Figs. 1–3. The curves in the figures are for illustration only, without any physical or theoretical justification.

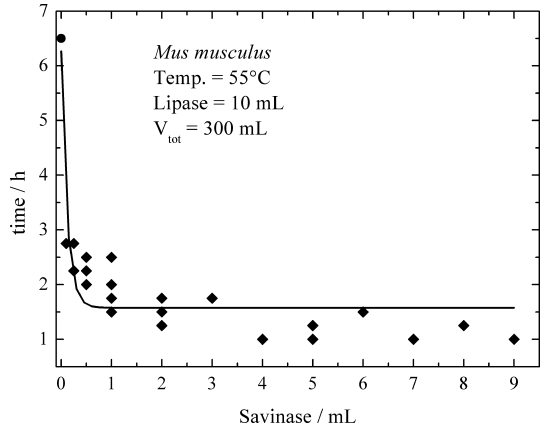

FIG. 1—*Maceration time in hours for mice as function of the amount of Savinase used. • marks the control with no protease added and ♦ marks points with protease added, the lowest amounts are 0.1 and 0.25 mL. The curve in the figure is for illustration only.*

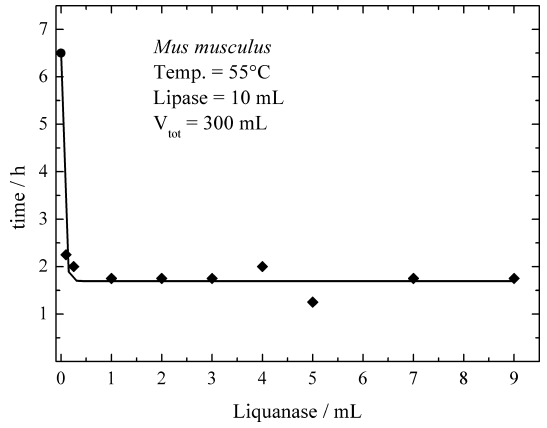

FIG. 2—*Maceration time in hours for mice as function of the amount of Liquanase used. • marks the control with no protease added and ♦ marks points with protease added, the lowest amounts are 0.1 and 0.25 mL. The curve in the figure is for illustration only.*

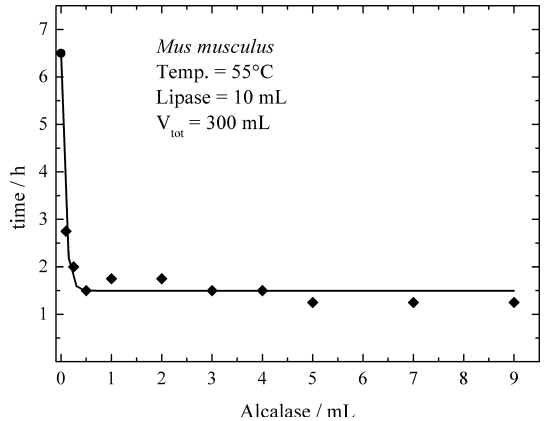

FIG. 3—*Maceration time in hours for mice as function of the amount of Alcalase used. • marks the control with no protease added and ♦ marks points with protease added, the lowest amounts are 0.1 and 0.25 mL. The curve in the figure is for illustration only.*

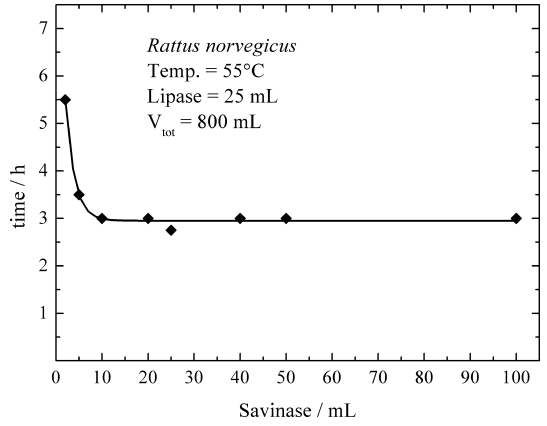

FIG. 4—*Maceration time in hours for rats as function of the amount of Savinase used. ♦ marks points with protease added, the lowest amounts are 2 mL, no control was made. The curve in the figure is for illustration only.*

As seen from the graphs, the speed of maceration clearly depends on the amount of protease used. However, it is also seen that the increase in speed of maceration saturates at ca. 4–5 mL and that further addition of the protease does not affect the time required for maceration. Similar to the experiments varying the amount of lipase, it was found that the use of a low amount of protease, <4–5 mL, resulted in greasy solutions, which were difficult to filter. For practical purposes, it is therefore recommended that an amount of 5 mL of protease is used for specimens of the size studied here.

In the series with Savinase, several double and triple experiments were performed. These demonstrated that the maceration time with the same amount of enzyme easily varied up to ½ h. The reason for this is unclear, but both variation in temperature and stirring speed, together with the uncertainty in the definition of the endpoint of the maceration time may be important.

The weight of each mouse after skinning and the mass of the dried bones after the maceration were measured in each experiment. The weight of the individual mice before skinning varied from 27.3 to 34.3 g and after skinning from 14.3 to 23.0 g. The weight of the macerated bones varied from 1.3 to 1.5 g, and the amount of flesh and fat removed by the enzymes was between 21.5 and 13.0 g. The average weight for 59 mice was 36.9 g before skinning and 18.4 g after skinning, and the average weight of the bones was 1.3 g giving an average amount of flesh and fat to be macerated of 16.9 g. However, we could not establish a correlation between the time of maceration and the amount of tissue to be macerated.

Maceration of Rats

To test the effectiveness of the enzymes on larger animals, a single experimental series using white WISTAR laboratory rats (*Rattus norvegicus*, gender: male) and Savinase® 16 L: Type EX was performed. The average weight of the rats was 271.7 g before skinning and 156.5 g after skinning, and the average weight of the bones was 12.3 g giving an average amount of tissue to be macerated of 143.2 g. All experiments were performed in a 1-L beaker, the total volume was 800 mL, the temperature was kept at 55 ± 5°C, and maximum stirring speed was used. A volume of 25 mL of Lipex® 100 L was chosen arbitrarily. The results are shown in Fig. 4.

As seen from the results in Fig. 4, enzymatic maceration works also very well for larger animals. As for the experiments on mice, the maceration time for rats depends on the amount of Savinase used. Also in this case, a lower limit for the maceration time

regardless of the amount of enzyme used is found. The use of 10 mL of Savinase gave a maceration time of 3 h that is the fastest time obtainable. This indicates that the surface area of the animal plays an important part in the maceration time together with the concentration of protease and the stirring of the maceration bath.

Influence of pH

The enzymes from Novozymes A/S are all products designed to be part of high-end detergents, and therefore, their optimum performance is developed to be in the basic pH region. The optimum performance of Savinase® 16 L, Type EX, and Lipex® 100 L is at pH = 9–11, even though they work over a very wide pH range. During the previous maceration experiments on mice and rats, the pH was measured to pH = 6 using simple universal pH paper test strips.

The influence of pH was tested on white rats to determine whether any notable reduction in the maceration time of 3 h could be obtained. The maceration bath consisted of 25 mL of Savinase® 16 L, Type EX, 25 mL of Lipex® 100 L, 700 mL of water, and 50 mL of 1 M buffer solution of ethanolamine/ethanolammonium hydrochloride. The pH in the bath was measured to 10 before the maceration commenced. During the maceration, the pH value changed and after ca. ½ h pH reached a value of 8 which remained constant during the rest of the experiment.

The maceration for rats using a basic buffer solution resulted in a maceration time of 2–2 ½ h which is a reduction compared to maceration in neutral solution. However, maceration in the basic buffer resulted in precipitation of a sticky brownish substance that adhered to the bones, making cleaning of the bones almost impossible. The brownish insoluble substance is likely to be calcium and magnesium salts of the free fatty acids generated by the enzymatic hydrolysis of the lipids. Even though the maceration time can be shortened by the use of basic maceration, the problem with formation and adherence of adipocerous material to the skeletal material makes this option nonviable in practice.

Appearance of the Macerated Skeletal Material

The visual appearance of the skeletal material from the enzyme maceration experiments was similar to bones prepared by traditional warm-water maceration. The distal and proximal parts of extremities were white to gray in color, whereas the diaphysis of the femur in particular had a reddish appearance. The visual difference most likely originates from the blood vessels and marrow in the bones because neither blood nor flesh was present on the surface of the bones.

To see whether the reddish color could be removed by enzyme treatment, some of the macerated femora were treated in pure protease solutions. Nine femora were placed individually in nine small beakers with 5 mL solution in each; Savinase® 16 L: Type EX was added to the first three beakers, Liquanase® 2.5 L to the next three, and Alcalase® 2.4 L FG to the final three. The solutions were then heated to 55 ± 5°C for 2 h. Color pictures were taken before and after the treatment, and a clear difference was recorded on all bones. The reddish color had almost disappeared even though the effect of Alcalase® 2.4 L FG seems less compared with the two other enzyme solutions. However, completely white bones are only obtained by treating the macerated material with dilute hydrogenperoxide solution (2–5%) for a couple of hours.

Discussion

The experimental results clearly show that the maceration time for mammals can be drastically reduced by the use of enzymes compared to the traditional warm-water maceration, which relies on bacterial decomposition of the flesh and soft tissues. The enzymatic maceration time for mice and rats is only 1–3 h in comparison with 4–7 days when warm-water maceration is used. The maceration of mice can be performed by the use of 1 mL of protease only. However, the use of 5 mL protease is recommended to avoid a greasy solution, which makes filtering of the maceration mixture very slow and necessitates repeated changing of the filter. The use of lipase in the enzyme maceration bath does not affect the time of the maceration, but lipase is needed to avoid a greasy solution. The use of 10 mL of lipase results in a solution of low viscosity, which makes filtering very easy.

In the case of rats, which in general have 10 times the amount of flesh and fat compared to mice, the amount of protease needed is the same or no more than twice as much. The use of 5 mL of Savinase® 16 L, Type EX resulted in a maceration time of 3.5 h and the use of 10 mL a maceration time of 3 h. In these experiments, the amount of lipase was arbitrarily chosen to be 25 mL.

The need for a more or less similar amount of protease when macerating a rat compared to a mouse indicates that the surface of the flesh and fat is a time-determining factor of the maceration, rather than the amount of soft tissue to be macerated because this is approximately 10 times higher for a rat than a mouse.

The other important time-depending factor is the stirring of the maceration bath. Fast stirring reduces the reaction time considerably: in experiments with mice, stirring resulted in a decrease in maceration time from 8 h down to 1 h only. This allows educational institutions, which normally have electric heaters with magnetic stirring to perform skeletal maceration within 1 day.

In addition to the greatly reduced maceration time, another notable difference is the absence of the obnoxious smell associated with warm-water maceration. This nauseating smell often makes it impossible to perform skeletal preparations at educational institutions, and for zoological museums, this requires investment in expensive ventilation systems.

The value and usefulness of enzyme maceration for large museums, which have the ventilation systems required for the traditional approach, depend on the number of animals to be macerated. For large series of specific mammals of 50 or more specimens, the maceration time itself is not the primary issue, even though the time can be lowered from several days to hours. In such cases, the time-consuming factor is the skinning of the animals, rinsing of the skeletal material, and registration of the individual animal. However, for small series or individual specimens, the method demonstrated here may also prove advantageous at museums.

According to their material safety data sheet, the concentration of enzyme in the commercial solutions from Novozymes A/S ranges from 1 to 10% (w/w) and the relative molar mass is ca. 30,000. Assuming the concentration is 5% and density of the solution is 1 g/mL, the amount of substance of enzyme using 5 mL of enzyme solution is 1.67 μmol and the concentration of protease in the 300 mL maceration bath is 5.5 μM only, which illustrates the effectiveness of modern enzymes.

The risk of working with enzymes is almost eliminated when they are supplied as solutions, because the dust problem associated with the use of enzymes in powder form is completely absent (11,25). The use of pure enzyme solutions instead of detergents for maceration avoids the presence of additives, such as builders (complexation agent), fluorescent whiteners, bleach, and corrosion inhibitors, which may affect the skeletal material and interfere with later analyses.

The color of enzyme-macerated bone is slightly reddish, which for some collection purposes might be unwanted. The traditional

whitish or yellowish color seen in most bone collections is achieved by treating the bones with a basic solution of hydrogen peroxide. By treating the enzyme-macerated bones in a similar way, similar appearances can be achieved. However, care should be taken when using this bleaching method because smaller bones tend to become brittle and fragile because of deterioration of the collagen matrix.

Conclusion

NMRI laboratory mice and white WISTAR laboratory rats were macerated by the use of enzymes, and the results show a dramatic reduction in maceration time (i.e., down to 1–3 h) compared to traditional warm-water maceration relying upon bacterial decomposition of the flesh and soft tissues which often requires 4–7 days. The time needed for the maceration is highly dependent on the stirring of the maceration bath and on the temperature of the bath. However, a working temperature of 55 ± 5°C is sufficient to obtain a fast maceration time and to avoid denaturation of the enzymes. The surface area of the mammals to be macerated is also an important parameter in the maceration time. For larger mammals, removing part of the flesh and soft tissue before maceration reduces the working time.

In addition to the reduced reaction time, the enzymatic method also eliminates the obnoxious smell associated with the traditional warm-water maceration method, making this method particularly useful in forensic science as well as for educational institutions and private conservators.

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