# ENZYMATIC DETECTION OF FORMALIN-FIXED MUSEUM SPECIMENS FOR DNA ANALYSIS AND ENZYMATIC MACERATION OF FORMALIN-FIXED SPECIMENS

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*Abstract*.—A simple enzymatic screening method has been developed to detect whether a tissue sample has been preserved with formalin or with ethanol only because such a method is a useful tool for predicting the quality of genetic test results. The method is based on enzymatic digestion at 55°C at neutral pH. The screening method shows that only ethanol-preserved tissue samples are dissolved, whereas formalin-preserved samples remain undissolved. The method was developed by the incorporation of laboratory rats preserved under controlled conditions in either 4% neutral buffered formalin or 96% ethanol. The method was subsequently tested on wild-living preserved specimens and an archived specimen. The protease enzyme used was Savinase® 16 L, Type EX from Novozymes A/S. The enzymatic screening test demands only simple laboratory equipment. The method is useful for natural history collections in museums where DNA analyses of archived specimens are performed. Wasted time and resources can be avoided through the detection of formalin-fixed specimens because these specimens yield low-quality, damaged DNA. In addition to the screening method, it is shown that formalin-preserved specimens can be macerated by enzymatic digestion under alkaline conditions at 55°C.

*Key words*.—DNA, enzymes, formalin fixation, maceration, screening method.

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## Introduction

Natural history and medical collections in museums and universities around the world represent an enormous archive of biological and genetic information on diversity, taxonomy, and diseases (Austin and Melville 2006, Burrell et al. 2015, Gilbert et al. 2007). As pressure on wild populations has increased over the past 200 years, the rare and extinct species found in museums have become increasingly valued as samples for genetic studies and representations of biological history (Austin and Melville 2006, Burrell et al. 2015). However, DNA extraction from archival specimens depends on their storage history, and it is widely known that formalin-preserved specimens give limited results for various reasons (Schander and Kenneth 2003, Austin and Melville 2006, Gilbert et al. 2007, Dietrich et al. 2013, Burrell et al. 2015). Treatment with formalin-containing solutions results in cross-links not only between proteins, but also between proteins and nucleic acids (Gilbert et al. 2007, Dietrich et al. 2013, Burrell et al. 2015). Additionally, cross-linking complicates isolation of DNA and introduces polymerase "blocks" during PCR (Gilbert et al. 2007). Formalin treatment also results in fragmentation of DNA, which can cause inhibition of the DNA polymerase, resulting in poor PCR amplification (Dietrich et al. 2013).

Formalin (aqueous formaldehyde solution) has been used for preservation and fixation of biological and medical materials since the early 1890s (Fox et al. 1985, Schander and Kenneth 2003). Formalin hardens the tissue through the formation of cross-links between amino acids in protein molecules (Fox et al. 1985, Schander and Kenneth 2003, Thavarajah et al. 2012). This fixation maintains body shape and increases specimen firmness, which is important for morphological studies (Hargrave et al. 2005). After the formalin preservation, museum specimens are often stored in an alcoholic solution, such as ethanol

(EtOH). For specimens recently collected and preserved by museum staff members, the preservation fluids used are known due to conservation reports. However, information regarding the preservation method of older specimens, donated specimens, or specimens collected in old uncatalogued lots are in many cases unknown due to lack of conservation data (Waller 1987). Furthermore, the concentration of formalin in EtOH in archived specimens may be below the detection limit for simple test kits, making these test kits unreliable (Waller 1987).

Reliable DNA information is easily accessible in specimens preserved in ethanol only and in specimens preserved before introduction of formalin in early 1890s. However, if a specimen is affected by formalin, it is necessary to know this prior to genetic analysis (Gilbert et al. 2007).

The aim of the present paper is to develop a simple method to assess whether museum specimens have been treated with formalin in order to save time and resources on DNA extraction from formalin-preserved specimens. The method used is a simple enzymatic test that does not require advanced laboratory equipment. In addition to this, a simple enzymatic method for preparation of skeletal material from formalin-fixed specimens is also developed.

## Experimental

### *Method*

In order to develop (1) a simple diagnostic enzymatic test for formalin preservation and (2) a method for cleaning formalin-fixed tissue from skeletal elements, we assembled a series of samples that were either fixed with formalin or treated with ethanol. For the first task we then tested whether proteases, at various concentrations, temperatures, and pH = 7, could digest the samples. Here the goal was finding conditions where formalin-fixed samples would fail to be digested, but non-formalin-fixed samples would be digested. As this method was meant to be a qualitative spot test, small tissue samples of $0.1 \times 0.1 \times 0.1$ cm were used. For the second task, we tested for pH conditions that would allow the digestion of formalin-fixed tissue. Since the purpose of this was to macerate the specimens, larger tissue samples with a size of $0.5 \times 0.5 \times 0.5$ cm were used. The enzymes used for development of the two methods were the protease Savinase® 16 L, Type EX because this is easily accessible and affordable. Details of our methods are found below.

### *Materials*

The following animals were used for the study: (1) Mammals: six different individuals of white male Wistar laboratory rats (*Rattus norvegicus*) and three polecats (*Mustela putorius*). (2) Reptile: one grass snake (*Natrix natrix*); these specimens were received in frozen condition. (3) Fish: one tub gurnard (*Trigla lucerne*); this specimen was an archived sample from the Natural History Museum of Denmark preserved in formalin in 1984 (T. Menne, pers. comm.) The 4% (v/v) neutral buffered formalin (NBF) was made by diluting 1 L of 40% (v/v) aqueous formaldehyde (stabilized with 10% methanol) in 9 L of deionized water followed by addition of 40 g $NaH_2PO_4 \cdot H_2O$ (0.29 mole) and 65 g $Na_2HPO_4$ (0.46 mole). (In some countries 4% NBF is referred to as 10% NBF because the concentrated stock solution at 40% [v/v] is called "100% formalin").

### *Enzymes*

The enzyme solution used was the protease Savinase 16 L, Type EX from Novozymes A/S. This commercial enzyme solution has a concentration of 1−10% according to the

Table 1. Dissolution of tissue samples from rats at 21°C and 55°C at pH = 7. Enzymatic dissolution of femur muscle tissue samples from laboratory rats preserved in either 96% EtOH or 4% NBF after treatment for 24 hours at pH = 7. Sample size = 0.1 × 0.1 × 0.1 cm. Volume of enzyme solution = 3 ml. Experiments were performed in triplicates with variable concentrations of Savinase at temperatures of 21°C and 55°C, respectively. C = enzyme concentration% (v/v), + = dissolution, and − = no dissolution.

| | T = 21°C | | T = 55°C | |
|---|---|---|---|---|
| C | EtOH | NBF | EtOH | NBF |
| 1 | − | − | + | − |
| 5 | − | − | + | − |
| 10 | − | − | + | − |
| 20 | − | − | + | − |
| 30 | − | − | + | − |

manufacturer. This enzyme solution was diluted with deionized water, and the various concentrations of Savinase used are given in percent (v/v) based on the commercial enzyme solution.

### *Sample Preparation*

Rats: After thawing, the rats were skinned and eviscerated. Three specimens were preserved in 10 L 4% NBF for 9 weeks and then stored in 96% EtOH. The other three specimens were preserved and stored in 96% EtOH only. Polecats: After thawing, the thighs were cut off and skinned. Three thighs were preserved in NBF and three in EtOH as described above. Grass snake: Samples were taken from the neck and tail part and preserved in either NBF (1 L) or EtOH as described above. Tissue samples for the enzymatic experiments from all specimens were sliced in pieces of either 0.1 × 0.1 × 0.1 cm or 0.5 × 0.5 × 0.5 cm. To achieve homogeneous samples from the rats and polecats, tissue was taken from the same location on the femur muscles.

### *Tests of Enzyme Concentration and Temperature*

All experiments were performed in triplicates with variable concentrations of Savinase at 21°C and 55°C, respectively. Three milliliters of a Savinase solution with a concentration between 1% and 30% were added to a 5 ml test tube. A tissue sample of 0.1 × 0.1 × 0.1 cm from EtOH- or NBF-preserved laboratory rats was added to each test tube. The tubes were then sealed with aluminum foil and placed at ambient temperature (21°C) or in an oven (55°C). The digestion of the tissue samples was visually evaluated after 24 hours (Table 1).

### *Verification of the Screening Method*

Based on the results from the experiments on laboratory rats, the screening method was tested on the wild-living specimens: the polecat, grass snake, and tub gurnard. The following experiments were performed in triplicates with 10% Savanase at 55°C and tissue samples preserved in EtOH or NBF, respectively. The size of the tissue samples was 0.1 × 0.1 × 0.1 cm for all specimens. The tub gurnard was preserved in NBF only. Experiments were performed as described above, and the digestion of the tissue samples was visually evaluated after 24 hours (Table 2).

### *Impact of Basic Conditions and High Temperature*

Solutions with and without the protease enzyme were tested to investigate the possibility of maceration of formalin-preserved specimens under alkaline conditions. The tissue

Table 2. Dissolution of tissue samples from wild-living specimens at 55°C and pH = 7. Enzymatic dissolution of tissue samples from three polecats (femur muscle) and one grass snake preserved in either 96% EtOH or 4% NBF. The tub gurnard tissue sample was from an archived and formalin-treated specimen from 1984. Samples were treated in 3 ml of 10% (v/v) Savinase solution for 24 hours at pH = 7. Sample size = 0.1 × 0.1 × 0.1 cm. Experiments were performed in triplicates. + = dissolution, − = no dissolution. NA = not available.

| Specimens and sample | EtOH | NBF |
|---|---|---|
| Polecat 1 | + | − |
| Polecat 2 | + | − |
| Polecat 3 | + | − |
| Grass snake neck part | + | − |
| Grass snake tail part | + | − |
| Tub gurnard | NA | − |

samples used had a size of 0.5 × 0.5 × 0.5 cm and were either EtOH or NBF preserved. Samples were taken from all specimens, and experiments were performed in triplicates. The samples were placed in 50 ml blue cap bottles, and we added either 20 ml of 10% Savinase solution + 0.19 g of $(NH_4)_2CO_3$ (2 mmole) or 20 ml of a solution of 0.1 M $(NH_4)_2CO_3$ only. The bottles were closed and placed at 55°C, and the digestion of the tissue samples was visually evaluated after 24 hours (Table 3).

## Results and Discussion

### *Impact of Enzyme Concentration and Temperature*

An overview of the various enzymatic digestion results is given in Tables 1–3. Table 1 shows that enzymatic digestion did not occur with EtOH-fixed or NBF-fixed rat tissue at ambient temperature (21°C) at pH = 7, even though the Savinase concentration was raised to 30%. However, when the temperature was raised to 55°C the EtOH-fixed tissue was completely dissolved, whereas the NBF-fixed tissue was still present and not degraded.

### *Verification of the Screening Method*

The results in Table 1 indicate that the temperature needed to be raised to 55°C before the enzymatic digestion of EtOH-preserved rat tissue occurred. Based on these results, the method was tested on tissue samples from some wild-living specimens. A concentration of 10% (v/v) of Savinase based on the commercial Savinase solution was arbitrarily chosen.

Table 3. Dissolution of tissue samples at 55°C and pH = 9. Dissolution of tissue samples after treatment with and without Savinase for 24 hours at 55°C and pH = 9. Specimens were preserved in either 96% EtOH or 4% NBF. Experiments were performed in triplicates. Sample size = 0.5 × 0.5 × 0.5 cm. Volume of solution = 20 ml. + = dissolution, − = no dissolution. NA = not available.

| | 10% Savinase + buffer | | Buffer only | |
|---|---|---|---|---|
| Specimens | EtOH | NBF | EtOH | NBF |
| Rat 1 | + | + | − | − |
| Rat 2 | + | + | − | − |
| Rat 3 | + | + | − | − |
| Polecat 1 | + | + | − | − |
| Polecat 2 | + | + | − | − |
| Polecat 3 | + | + | − | − |
| Grass snake neck part | + (−skin) | + (−skin) | − | − |
| Grass snake tail part | + (−skin) | + (−skin) | − | − |
| Tub gurnard | NA | + | NA | − |

As seen in Table 2, all tissue samples from specimens preserved in EtOH were completely dissolved, whereas samples from NBF-fixed tissue remained undegraded. The archived fish samples, which were preserved in formalin in 1984, showed the same result; none of the samples were dissolved after 24 hours, though the enzyme solution became yellowish. This coloration could be a sign of tissue changing after an extended storage period.

### *Preparation of Skeletal Material from Formalin-Fixed Specimens*

The enzyme Savinase 16 L, Type EX used in this work is developed for detergent use, and its optimum effectiveness is designed to be in the basic pH region (pH = 9−11) with a maximum temperature performance at 55°C (Simonsen et al. 2011, Uhre et al. 2015). To investigate whether enzymatic maceration of formalin-preserved specimens was possible, experiments were performed at alkaline conditions. Ammonium carbonate was used as a buffer, because this salt essentially forms an ammonium-ammonia buffer with pH = 9.2 (Wen and Brooker 1995). Solutions of ammonium carbonate only were included in this experimental series to investigate whether a basic solution alone was able to cause dissolution of EtOH- or NBF-preserved tissue samples. Since the purpose of this experimental series was to macerate the specimens, larger samples with a size of $0.5 \times 0.5 \times 0.5$ cm were used. For the grass snake, part of the skin was present on the samples.

As seen in Table 3, only solutions containing enzymes resulted in complete dissolution of the tissue samples. In the solutions with ammonium carbonate only, the tissue samples were still present. However, the EtOH-preserved samples were slightly affected on the surface and the formalin-preserved samples were a bit swollen, though otherwise unaffected. This shows that high temperature and alkaline pH had some impact on the tissue, but much less compared to the impact of the enzymes.

Also worth noting is that skin from the grass snake was undigested. This is most likely the result of the keratin material of epidermis being different from muscle tissue. Keratin is a stiff structural protein extensively cross-linked by intermolecular disulphide bonds (-S-S-) between the thiol groups of cysteine residues (Alibardi and Toni 2005, Swadźba et al. 2009). As Savinase hydrolyzes only the peptide bonds in proteins and does not exhibit disulphide reduction properties, the keratin part is left undigested (Letourneau et al. 1998, Yamamura et al. 2002).

## Conclusion

A simple screening method for detection of formalin-preserved tissue has been developed through the use of tissue samples from laboratory rats preserved under controlled conditions in either 96% EtOH or 4% NBF. It was shown that at neutral pH and at ambient temperature neither EtOH- nor formalin-preserved tissue could be dissolved by the protease enzyme Savinase 16 L, Type EX from Novozymes A/S. However, by performing the reaction at 55°C, it was shown that the EtOH-preserved tissue samples completely dissolved, whereas the formalin-preserved samples remained undegraded and present in the enzyme solution. The developed method was subsequently tested on wild-living and archived specimens that were either EtOH- or formalin-fixed. These experiments showed the same results as those for the laboratory rats. Only the EtOH-preserved tissue samples were dissolved by the enzymes, while the formalin-preserved samples were undigested.

This developed procedure is simple and does not require advanced laboratory equipment. The procedure is useful for museums with natural history collections where DNA analyses of archived species are performed. Detection of formalin-fixed tissue saves both time and resources spent on DNA extraction from formalin-preserved specimens.

The impact of high-temperature and alkaline conditions, both with and without the presence of protease enzyme, was also investigated. This showed that the tissue samples were almost unaffected by a solution being alkaline only. However, when the reaction was performed in a solution containing enzymes and being alkaline too, it was shown that enzymatic maceration could be achieved. This method is useful if preparation of skeletal materials is needed from formalin-preserved specimens.

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