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Fast, gentle and inexpensive maceration of teleostean bones using Enzyrim

Schnelle, schonende und kostengünstige Mazeration von Teleostierknochen mit Enzyrim

Lisa Klemens[1] & Timo Moritz[1,2]
[1] Deutsches Meeresmuseum, Katharinenberg 14-20, 18439 Stralsund, Germany
[2] Institute of Zoology and Evolutionary Research, Friedrich-Schiller-University Jena, Erbertstr. 1, 07743 Jena, Germany, timo.moritz@outlook.com

Summary: Bones, especially teleostean bones, are used for species identification e.g. in diet and paleontological analyses. For this purpose, reference collections of bones are more useful than pictured identification indexes. Maceration enables long term storage of bones. Most maceration methods like cooking or using commercial detergents are quite time intense and cause damage to the objects, like exposition of porous bone structure or loss of thin lamellar bone. We discuss different methods and report a simplified and cheap protocol for the use of the enzyme solution Enzyrim OSS. We recommend working with 1-litre volumes, using tap water without pH adjustment, and adding Supralan depending on the fat content of a specimen. Thus, optimal results with low costs are obtained.

Key words: Osteology, fish bones, Supralan, enzyme

Zusammenfassung: Knochen, besonders die der Teleostei, werden u.a. in Nahrungsanalysen und paläontologischen Untersuchungen für Artbestimmungen verwendet. Hierfür ist eine Referenzsammlung der Knochen oft hilfreicher als ein bebilderter Bestimmungsschlüssel. Mazeration ermöglicht eine langfristige Aufbewahrung der Knochen. Die meisten Mazerationsmethoden wie Abkochen oder das Verwenden von Waschmitteln sind jedoch sehr zeitintensiv und schädlich, da sie poröses Knochengewebe freilegen oder dünne Knochenteile zerstören. Wir besprechen verschiedene Methoden und geben hier ein vereinfachtes und günstiges Protokoll für die Anwendung der Enzymlösung Enzyrim OSS wieder. Wir empfehlen, mit 1-Liter-Volumina zu arbeiten, Leitungswasser zu verwenden, ohne den pH-Wert anzupassen, und Supralan entsprechend dem Fettgehalt der Exemplare zuzugeben. Auf diese Weise erzielt man optimale Ergebnisse zu niedrigen Kosten.

Schlüsselwörter: Osteologie, Fischknochen, Supralan, Enzym

1. Introduction

The bones of teleosteans are a vital part of taxonomical identification, especially in paleontology, archaeology or food-web ecology studies (e.g. Fitch & Brownell 1968; Jobling & Breiby 1986; Pierce & Boyle 1991; Campana 2004). Taxonomical identification with established identification guides, like Härkönen (1986), Leopold (2001), Campana (2004) or Tuset et al. (2008), however, have limitations due to the two dimensionality of pictures and not consistent nomenclature of specific bone structures. Hence a collection of reference bones is useful since it can overcome this limitation.

By removing flesh and fat maceration enables a long-lasting preservation of bones. Teleostean bones can be cleaned with different established maceration methods like cooking, warm-water maceration or with various commercial detergents (Mooney et al. 1982, Bartels et al. 1992, Grundmann & Rötzscher 1999, Steadman et al. 2006, Simonsen et al. 2011). Independently of the size of the fish most of these methods

are quite work intensive and time consuming. Furthermore, some bones, like cleithrum, preoperculum or lacrimal, are quite fragile or have at least fragile parts. Heat, aggressive detergents or additional bleaching usually damages the cortical structure of the bone, exposing the porous interior of bones, thus increasing the risk of destroying parts of the respective skeletal structures (Couse & Conner 2015). Using Enzyrim OSS forte (Bauer Handels GmbH, Adetswill, Switzerland) can overcome several of the above-mentioned shortcomings (Grundmann & Rötzscher 1999, Postl et al. 2008). We report here an optimized maceration protocol to get isolated teleostean bones in a reasonable timespan, in high quality and at low costs and compare the results with the traditional cooking method.

2. Material and Methods

For our procedure we used fresh-thawed fish. Six freshwater fish species and eight saltwater fish species of sizes between 8 cm and 110 cm standard length (Tab. 1) were macerated with Enzyrim. For comparative reasons equally sized specimens of *Cyprinus carpio*, *Squalius cephalus*, *Gadus morhua*, *Merlangius merlangus* and *Sander lucioperca* have been 'traditionally' mazerated by cooking, but also with the use of Supralan as below described for the Enzyrim protocol. Additional isolated bones obtained via traditional cooking method (without Supralan) where available for comparison of results.

Before the actual maceration processes excess, meat and intestines were roughly removed by hand of each specimen. The optimal working

Tab. 1: Tested specimens in this study. SL_{enz} – standard length of Enzyrim specimen in millimeters; h – maceration duration in hours; SL_{co} – standard length of cooking-method specimen in millimeters.
Tab. 1: In dieser Untersuchung verwendete Art. SL_{enz} – Standardlänge der Enzyrimexemplare in Millimetern; h – Mazerationsdauer in Stunden; SL_{co} – Standardlänge der gekochten Exemplare in Millimetern.

Taxon	SL_{enz}	h	SL_{co}
Cyprinidae			
Carassius carassius	310	5	–
Cyprinus carpio	309	5	320
Squalius cephalus	230	4	238
Tinca tinca	178	4	–
Osmeridae			
Osmerus eperlanus	146; 152; 185	2	–
Gadidae			
Gadus morhua	275	2.5	293
Merlangius merlangus	29.8	2	33.5
Lotidae			
Molva molva	1100	3.5*	–
Percidae			
Sander lucioperca	285	4	265
Labridae			
Ctenolabrus rupestris	87	3	–
Symphodus melops	75; 85; 87	2	–
Callionymidae			
Callionymus lyra	248; 363	4	–
Sebastiidae			
Sebastes viviparus	160; 165	2.5	–
Caproidae			
Capros aper	135	2	–

*) Macerated in four portions; each portion took 3.5 hours for maceration, but could have been done in parallel.

temperature of Enzyrim is between 55 and 60 °C. Therefore, tap water needs to be heated to about 56 °C before adding Enzyrim and kept by this temperature with a heating plate. Very useful for this step are temperature adjustable magnetic stirrer as present in many laboratories (don't use the stirring function). After heating add 10 ml Enzyrim and 20 ml Supralan 67 (Bauer Handels GmbH, Adetswill, Switzerland) per liter. For big specimens larger volumes may be used, but most reasonable is the use of only 1-litre volumes to keep best control over the maceration progress and to avoid loss of small pieces. It can also be an option to process larger specimens in parts, as tested here for a 110 mm SL *Molva molva*. Very fatty specimens require a higher amount of Supralan, e.g. for larger cyprinids we doubled the amount to 40 ml per liter

After 2 to 5 hours most flesh is dissolved and the specimen respectively its parts can be taken out of the solution. When still moist, spinal cord and small remaining pieces of flesh are carefully removed by hand with tweezers. Bones are then placed on a flat plastic surface to dry for several days at room temperature. When sufficiently dried there will be no problem with mold or smell. An additional bleaching step is not necessary.

3. Results and discussion

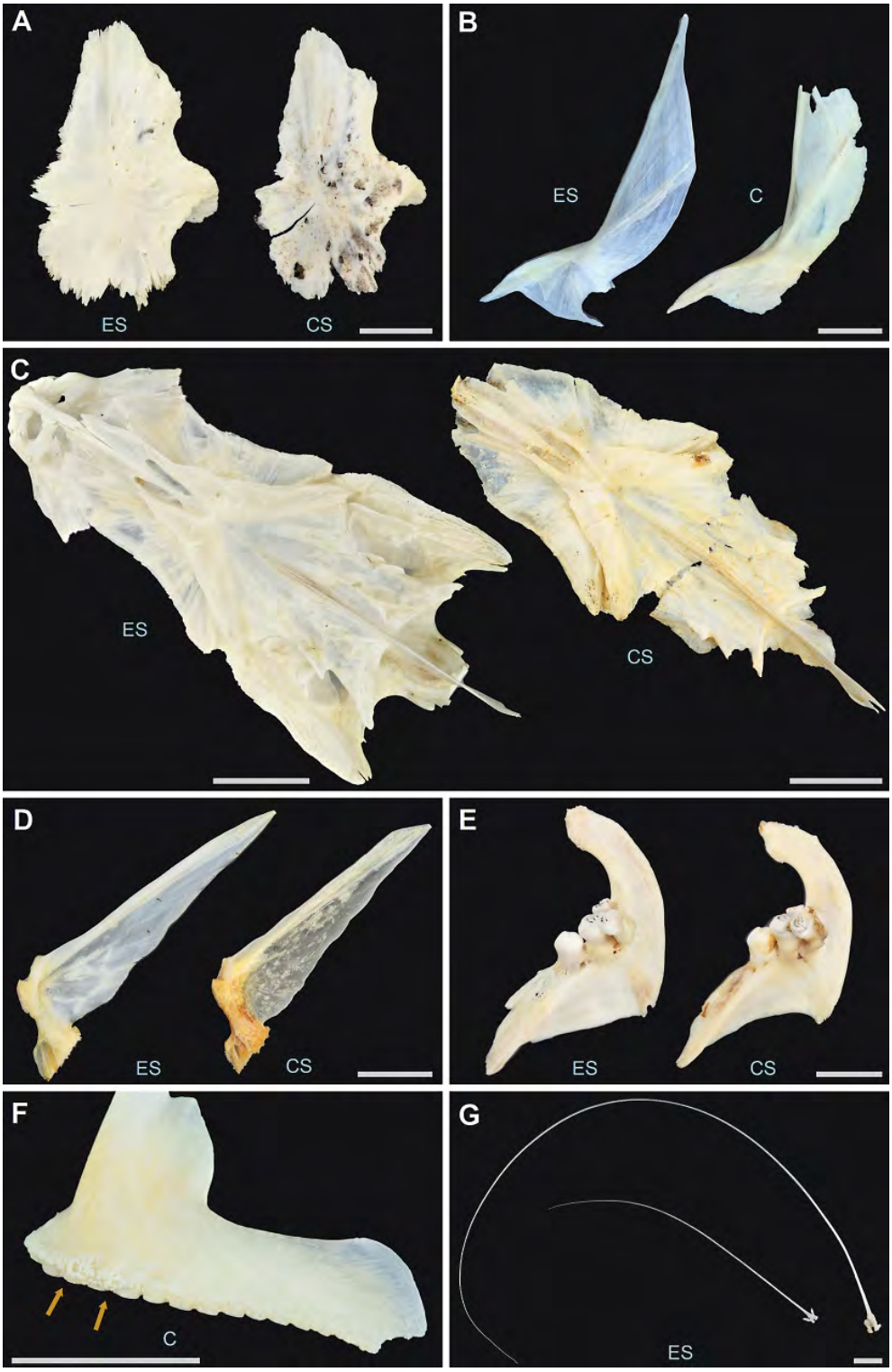
Maceration with Enzyrim, without adjusting of pH-value and without using bleaching detergents, is a fast and gentle approach. Especially in comparison to maceration methods which involve cooking, the advantages become visible (Fig. 1). Heat or components of commercial detergents, like bleach or leach, damage the cortical tissue of bones and therefore destroy the bone structure (Grundmann & Rötzscher 1999, Steadman et al. 2006, Postl et al. 2008, Simonsen et al. 2011, Couse & Conner 2015). Damage of the outer bone layer exposes the internally porous structure. Thin and porous parts of the bones often break apart while cooking or during successive handling (Fig. 1A-D). Furthermore, by the simple boiling method, which is still commonly used, most flesh has to be removed by hand, making this a rather time-consuming method.

Specific enzymes do not react with bony tissue and the low optimal temperature of Enzyrim between 55-60 °C is not damaging the cortical tissue. Therefore, maceration with Enzyrim does not cause damage to the bone, allowing also e.g. morphometric studies of single bones (e.g. Riedlecker & Herler 2009). Even multi-bone components like the neurocranium more often remain connected to each other (Fig. 1C), and very delicate structures like thin fin rays can be obtained in perfect shape (Fig. 1G).

Enzyrim itself will not cause damage to DNA, as it can even be used to improve DNA extraction (Mályusz et al. 2006). Therefore, the herein described protocol should be not totally denaturate DNA, thus even allowing future DNA analyses (Steadman et al. 2006, Li & Liriano 2011).

Compared with other methods, the Enzyrim approach is quite fast. The complete maceration process as described above using Enzyrim and Supralan takes between 2 to 5 hours depending on the size and fat content of the fish. Smaller specimens, e.g. 8 cm *Symphodus melops*, and fish with moderate and body fat content, e.g. 18.5 cm *Osmerus eperlanus*, are usually done within a maceration time of only about 2 hours (Tab. 1). Fish with a high body fat content like cyprinids took a longer maceration time, but this again can be shortened by adding a higher amount of Supralan.

The duration of other established maceration methods, like cooking with Supralan alone or warm-water maceration, take much longer (Mooney et al. 1982, Grundmann & Rötzscher 1999, Steadman et al. 2006, Simonsen et al. 2011). Furthermore, even after treating bones with these maceration methods for a long period of time, the bones are still stained and not completely cleaned of flesh. A lot of remaining tissue pieces need to be removed by hand, which is laboriously and a difficult task because of the exposed porous bone structure caused by the heat (Fig. 1F). Bones treated with Enzyrim are well cleaned after the maceration process and need only little past processing: only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the spinal cord and little scraps of flesh need to be removed by hand.

The duration of the maceration process seems not to be very sensitive on the pH value. Grundmann & Rötzscher (1999), Postl et al. (2008) and the producer Bauer recommend using a pH value of 8.5. Following the product information sheet Enzyrim is stable at pH values between 4 and 9 (Sicherheitsblatt Enzyrim OSS; Bauer Handels GmbH, Adetswill, Switzerland). The used tap water had a pH value around 7.2, so in order to reduce resources, work and costs we did not adjust the pH value. Nevertheless, the maceration process was very fast and the bones were perfectly macerated.

There are also other enzymes which could be used for enzymatic maceration, like trypsin. To be able to macerate with trypsin the bones need to be preprocessed over several hours. Furthermore, this method includes chemicals like potassium hydroxide (KOH) or Ammonia (NH_3) which makes this method more time consuming and more dangerous (Piechocki & Altner 1998; Postl et al. 2008; Li & Liriano 2011). Trypsin from abattoir refuse is no longer available and biotechnically produced trypsin is rather cost intensive for an unspecific maceration process.

Bioenzym SE (Spinnrad, Bad Segeberg, Germany) is another enzymatic detergent used for maceration. However, working with Bioenzym SE is more expensive. For maceration with Bioenzym SE a 10% enzymatic solution is commonly used (Bartels et al. 1992). In comparison with Enzyrim only few milliliters are needed for the maceration solution. Using 10 ml of Enzyrim in a 1 liter maceration solution costs 0.63 € (Bauer Handels GmbH, Adetswill, Switzerland), while using a 10% Bioenzym SE maceration solution costs 1.08 € (Spinnrad, Bad Segeberg, Germany). Also, maceration with Bioenzym SE is more time consuming: 20 h were needed to macerate only the head of trout and pike (Bartels et al. 1992).

In addition to Enzyrim we used Supralan to fasten and enhance the maceration process by degreasing. This rather cheap detergent will increase the costs per liter of maceration solution for another 0.11 €. We don't recommend using an additional lipase like Simonsen et al. (2011) did, as lipases are not as efficient as Supralan and leave a greasy filtrate. Simonsen et al. (2011) in their study described a brownish stain on the bones after using Enzyrim and recommended bleaching. However, after the maceration process as described herein the bones are perfectly

Fig. 1: Skeletal elements of teleosts after maceration process. **A** Right frontal of *Cyprinus carpio*, dorsal view, 320 mm SL [DMM IE/16626] (left) and 309 mm SL (right). **B** Left cleithrum of *Merlangius merlangus*, lateral view, 298 mm SL [DMM IE/16619] (left) and *Trisopterus luscus* 278 mm SL [DMM IE/16600] (right). **C** *Gadus morhua* neurocranium, dorsal view, 275 mm SL [DMM IE/16628] (left), and detached frontal, dorsal view, 293 mm SL (right). **D** Left pelvic bone of *Zander lucioperca*, dorsal view, 285 mm SL (left) and 265 mm SL [DMM IE/16662] (right). **E** Left fifth ceratobranchial of *Squalius cephalus* 230 mm SL (left) and 238 mm SL [DMM IE/16623] (right). **F** Left premaxillary of *Pleuronectes platessa*, lateral view, 270 mm SL [DMM IE/16601], arrows point to areas, where the cortical tissue of the bone is damaged and the interior porous structure exposed. **G** Elongated fin rays of *Callionymus lyra,* 363 mm SL [DMM IE/16627]. Blue letters indicate maceration method: C cooking; CS cooking plus Supralan; ES Enzyrim plus Supralan. All scale bars 10 mm.
Abb. 1: Knochenelemente von Teleostiern nach dem Mazerationsvorgang. **A** Rechtes Frontale von *Cyprinus carpio*, von dorsal, 320 mm SL [DMM IE/16626] (links) und 309 mm SL (rechts). **B** Linkes Cleithrum von *Merlangius merlangus*, von lateral, 298 mm SL [DMM IE/16619] (links), und *Trisopterus luscus,* 278 mm SL [DMM IE/16600] (rechts). **C** *Gadus morhua*, Neurocranium, von dorsal, 275 mm SL [DMM IE/16628] (links), und abgelöstes Frontale, von dorsal, 293 mm SL (rechts). **D** Linker Beckenknochen von *Zander lucioperca*, von dorsal, 285 mm SL (links) and 265 mm SL [DMM IE/16662] (rechts). **E** Linkes fünftes Ceratobranchiale von *Squalius cephalus,* 230 mm SL (links) und 238 mm SL [DMM IE/16623] (rechts). **F** Linkes Prämaxillare von *Pleuronectes platessa*, von lateral, 270 mm SL [DMM IE/16601], Pfeile zeigen auf Bereiche, in denen die corticale Schicht beschädigt und so die interne poröse Schicht exponiert wurde. **G** Verlängerte Flossenstrahlen von *Callionymus lyra,* 363 mm SL [DMM IE/16627]. Blaue Buchstaben geben die Mazerationsmethode an: C Kochen; CS Kochen plus Supralan; ES Enzyrim plus Supralan. Alle Maßstäbe 10 mm.

white and quite odorless and don‘t need additional bleaching.

In summary, we conclude that the herein described procedure for the maceration of teleostean bones is excellent when comparing costs, time requirement and quality of results.

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