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New techniques for the recovery of small amounts of mature enamel proteins

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ABSTRACT

Currently there are no non-destructive techniques to obtain protein from the dental enamel, the most mineralized tissue in mammals and most resistant to diagenesis, which provides a window to the developing period by means of incremental markings containing proteins. To recover protein, dissolution of powdered enamel is required. Here we tested whether samples obtained by micro-etching of the enamel surface were adequate for protein analysis by MALDI-TOF/TOF mass spectrometry and identification in protein databases. The micro-etch techniques were effective in generating adequate samples for mass spectrometry (from 3 to 13.4 μm superficial enamel), being also highly conservative, since they rendered masses of enamel ranging from 0.1 to 0.4 mg. Using these techniques the separation of proteins by SDS-PAGE was not necessary, and the whole procedure was easier. Results showed successful identification of specific enamel proteins after whole crown superficial etching with 11% EDTA in the case of immature porcine samples, and with 10% HCl in the case of mature human enamel. X- and Y-isoforms of amelogenin, ameloblastin, and enamelin peptides were identified. The new techniques described here allowed the successful recovery of enamel proteins, opening new avenues for the use of enamel protein information in fossil/archeological material, where sometimes little protein is left.

1. Introduction

Dental enamel contains 95% of mineral by weight, being the most highly mineralized tissue in the body. It also contains a small amount of protein (~2%), and most of these are specific to the enamel, such as amelogenins (Snead et al., 1985), ameloblastin (Krebsbach et al., 1996) and enamelin (Fukae et al., 1996). Amelogenins are a heterogeneous group of proteins, and a specific amelogenin sequence is derived from the X- and another from the Y-chromosome (Pfeiffer and Brenig, 2005; Nielsen-Marsh et al., 2009). The fact that enamel has this high amount of mineral turns it into a matrix that is highly inert to changes brought about by time and the environment (Lee-Thorp and Sponheimer, 2006).

Recently, peptides derived from enamel proteins have been successfully recovered from contemporary, medieval, and two Neanderthal teeth (Nielsen-Marsh et al., 2009). In the latter case (Nielsen-Marsh et al., 2009), protein extraction from the tooth enamel was accomplished from ~2 mg of enamel powder from each of the Neanderthalis teeth as the starting material, and the precipitation protocol was based on the use of TCA (Nielsen-Marsh et al., 2009), as proposed for the recovery of proteins from mature rodent enamel (Porto et al., 2006). An older study also used acid dissolution to extract and characterize mature bovine enamel proteins. However, the starting material was large (seven thousand teeth!) and the results reported no specific peptide sequences, but rather the most abundant amino acids found (Glimcher and Levine, 1966).

Although enamel protein recovery is challenging, the fact that these proteins are specific to enamel is advantageous in the case of ancient and forensic material, in which contamination with proteins from the lab (glassware, gloves, etc) or from the environment is a problem.

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The recent analyses of organic matter in enamel (Sousa et al., 2006) show that the amount of organic matter decreases from the surface of the enamel toward the more inner layers up to ~600 μm in occlusal enamel (Sousa, 2009, personal communication). Thus, the superficial dental enamel may be advantageous for protein analysis because it may contain more protein and is more easily accessible. Moreover, superficial etching techniques allow researchers to obtain enamel samples without the need for using complex procedures to separate dentin from the enamel. As a matter of fact, we have been using a 20-s superficial enamel acid etching technique to collect superficial enamel samples for lead analysis (Gomes et al., 2004; Costa de Almeida et al., 2007; de Almeida et al., 2008; Costa de Almeida et al., 2009), since lead and other micro-elements such as fluoride, zinc, and strontium accumulate in this outer layer of the enamel (de Souza Guerra et al., 2010).

Our hypothesis in the start of this study was that we could obtain enough protein for protein analysis using superficial enamel etching techniques. Thus, the aim of this study was finding a method to obtain superficial enamel samples for protein analysis from mature dental enamel, and testing whether the samples were adequate for mass spectrometry and protein fingerprinting.

2. Materials and methods

2.1. Enamel samples

Developing porcine incisors were collected from two-month-old male pigs that were killed in local slaughterhouses. The incisors were washed with distilled water, to clean the tooth surface. Teeth were kept at −20 °C until use. This study was approved by the Institutional Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). Human third molars were extracted in the Oral Surgery Clinic of the Dental School of Ribeirao Preto, SP, Brazil. The patients were informed both verbally and in writing about the purposes of the research, and signed an informed consent document and a tooth donation term. Teeth were stored at −20 °C until processing.

Porcine teeth were selected for this study because they were not fully mature, so it was expected that it would be less difficult to recover enamel-specific proteins from them. The use of protease inhibitors throughout this study was essential to avoid proteolysis, and the inhibitors employed here were Phenylsulfonylfluoride, N-Ethylmaleymide, and Phenanthroline, all purchased from Sigma–Aldrich (St Louis, MO, USA) as powders, and prepared as stock solutions in methanol, being diluted in buffer solution at the appropriate concentration (2 mM for all) just prior to use.

2.2. Protein extraction techniques

We tested 3 techniques to obtain enamel samples: a) enamel powder dissolution followed by TCA precipitation; b) whole crown etching with different acids; and c) restricted area etching with different acids.

2.2.1. Enamel powder dissolution followed by precipitation with Trichloroacetic acid (TCA)

The crowns of human third molars ($n = 4$) were powdered in a cryogenic mill Model MA 775 (Marconi, Piracicaba, SP, Brazil), using liquid nitrogen. The dentine was separated from the enamel by a classic density gradient fractionation procedure (1) using a 91% bromophorm/9% acetone mixture. After separation, the enamel and dentin powders were dried in a lyophilizer. Some samples were applied to glass slides and checked under a polarizing light microscope, confirming the identity of enamel and dentin, and the complete separation of the two tissues. The enamel powder was dissolved in 12% trichloroacetic acid (TCA) in a proportion of 200 μL acid/mg enamel powder. One hour later, sodium deoxycolate was added in a final concentration of 200 μg/mL, as described earlier (Porto et al., 2006). After enamel dissolution at 0 °C, the solution was centrifuged at 2500 × g at 4 °C for 45 min. The pellet was ressuspended in 200 μL of a 6 M urea solution containing a wide range of protease inhibitors, as described before (Porto et al., 2006).

2.2.2. Whole crown etching

Porcine teeth (which were not fully mature; $n = 2$) were only etched using ethylenediamine tetraacetic acid (EDTA), which is the most gentle acid solution routinely used to decalcify tissues in Histology labs. This procedure resulted in adequate amounts of proteins for the identification of specific peptides from enamel protein after MALDI-TOF/TOF-MS analysis.

At first, mature human third molars ($n = 20$) were also etched with various concentrations of EDTA using different times: 15, 20, and 30 min, and 4 and 16 h, with 4 teeth for each dissolution time. However, no peptide signal that resulted in enamel-specific protein could be retrieved, so stronger acids were tested. Hydrochloric acid (HCl) resulted in an appropriate amount of dissolved enamel to retrieve enamel-specific proteins. TCA was also used to etch samples. Various etching times were tested for 10% HCl (1, 2, and 5 min; each one with 4 teeth) and for 12% TCA (5, 15, and 30 min, each one with 4 teeth).

2.2.3. Restricted area etching

This etching technique was tested in both porcine (immature enamel; $n = 2$) and human teeth (mature enamel, $n = 4$), and allowed the sampling of enamel from a specific area "restricted" by a circular hole in a tape. An important note: glycerol and other agents may be used to change the superficial tension of the solution, resulting in acid attacks that are less invasive.

Briefly, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the teeth. A window of 3.14 mm^2 area was exposed, and 5 μL 11.2% EDTA/1.25% NaOH, pH 7.3, were applied for 15 s. The extract was transferred to a low protein binding tube. This etching step was repeated twice, and the etching solutions were added to the tube. At the end, 5 μL of distilled water were applied to the tape window for 5 s and added to the same tube, which contained approximately 20 μL water.

We usually use glycerol (70%, v/v) to obtain clinical samples from deciduous teeth in vivo (Fukae et al., 1996; Krebsbach et al., 1996; Pfeiffer and Brenig, 2005; Nielsen-Marsh et al., 2009). However, in this study glycerol was not employed in the etching solutions because it interferes in the drying of the MALDI/TOF matrix, and should therefore be avoided in samples intended for mass spectrometry.

2.3. Determination of phosphorus

Phosphorus was determined by the colorimetric method of Fiske and Subbarow (1925) (Fiske and Subbarow, 1925), as described in Costa de Almeida et al. (2007). Assuming an enamel density of 2.95 g cm^{-3} and a phosphorus content of 17.4% (Koo and Cury, 1998), and accepting an average presumed area of the tooth crowns used, the presumed etched depths using the different acid solutions were calculated. Phosphorus determination was also useful to quantify the sample masses obtained by the whole crown and the restricted area etching techniques (since they dissolve the enamel, and therefore the initial mass cannot be weighed).

2.4. Electrophoresis and digestion of proteins from gel bands

Protein solutions from the porcine teeth obtained by the restricted area etching procedure and protein solutions from the