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Recovery and identification of mature enamel proteins in ancient teeth

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Proteins in mineralized tissues provide a window to the past, and dental enamel is peculiar in being highly resistant to diagenesis and providing information on a very narrow window of time, such as the developing period; however, to date, complete proteins have not been extracted successfully from ancient teeth. In this work we tested the ability of a whole-crown micro-etch technique to obtain enamel protein samples from mature enamel of recently extracted ($n = 2$) and ancient ($n = 2$; AD 800 to 1100) third molars. Samples were analyzed using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry, and the resulting spectra were searched against the Swiss-Prot protein database using the Mascot software for protein identification. In our protocol, the separation of proteins in gel is not necessary. Successful identification of specific enamel proteins was obtained after whole-crown superficial enamel etching with 10% HCl. Most protein fragments recovered from dry teeth and mummy teeth contained amino-terminal amelogenin peptides. Only one peptide specific for the amelogenin X-isoform was identified. In conclusion, the reported techniques allowed the successful recovery of proteins specific to dental enamel from samples obtained in a very conservative manner, which may also be important in forensic and/or archeological science.

Mature dental enamel is the most mineralized tissue in mammals, containing 95% mineral (weight %), < 1% organic matter, and an even smaller amount of proteins, most of which are specific to the dental enamel (1). In comparison, compact bone is a mineralized tissue that contains 70% mineral (by weight) and around 28% of collagen type I (2), the most abundant protein in vertebrates (3).

As a result of its characteristics, enamel is highly inert to changes brought about by time and the environment, and presents the greatest resistance to diagenesis (chemical changes after death), and is thus a very important source of information for palaeologists, palaeanthropologists, and anthropologists. Indeed, these professionals use the morphological aspects of enamel to infer data on the type of food ingested. In addition, the histological aspects of enamel can provide useful information on many different physiological aspects of past species (4, 5). As recently stated, much of what is known about (human and primate) evolution derives from dental remains (6).

Proteins are the building blocks and the machinery of organisms, and because of their different levels of expression, alternative splicing, and many possible post-translational modifications (3), they are not directly related to the information conveyed in the DNA. Therefore, the search for information on proteins from past species is extremely important.

In this regard, an amazing achievement has been the publication of mass spectrometry (MS) data from bone extracts of an 80-million-year-old dinosaur (*Brachylophosaurus canadensis*) (7), describing the identification of eight collagen peptide sequences, amounting to 149 amino acids of the collagen sequence. These collagen peptides were shown to contain OH-proline amino acids (a post-translationally modified amino acid). The hypothesis that endogenous proteins can persist across geological time is still controversial, but data support the likelihood that protein remnants are preserved when buried deeply in sandstone (8). There are also theories that point to the importance of the 'packaging' of mineral-bound biopolymers in bones for the long-term survival of proteins (9). A case in point is osteocalcin, a small protein that is strongly bound to collagen and mineral (9). The complete sequence of the osteocalcin protein (5.5 kDa) obtained from small amounts (20 mg) of two bison bones older than 55000 yr has been reported (10). In 2005, osteocalcin

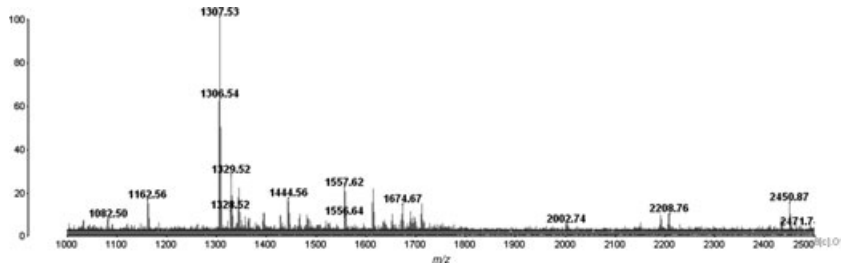

Fig. 1. Mass spectrum of a superficial enamel extract of a contemporary mature human molar obtained by whole-crown etching in 10% HCl for 5 min. Two amelogenin X-isoform peptides were identified [with mass-to-charge ratio (m/z) peaks of 1307.53 and 1557.62] (protein score: 59). Technical parameters of the spectral information obtained are: 15 mV [sum = 990 mV] profiles 1–64 smooth Gauss 2-baseline 5.

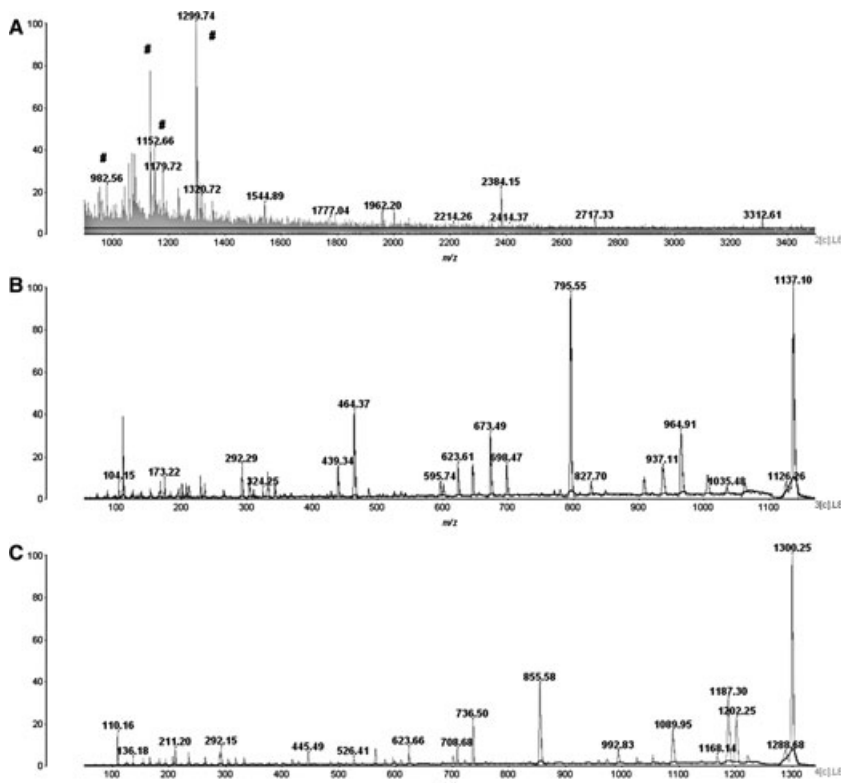

Fig. 2. (A) Mass spectrum of a superficial enamel extract of a contemporary human molar (that was dry at the time of analysis). This extract was obtained by whole-crown etching in 10% HCl for 5 min. Five amelogenin peptides were identified (protein score: 216 for amelogenin Y and 211 for amelogenin X). Ions identified as amelogenin. (B) Mass spectrum of collision-induced dissociation mass spectrometry/mass spectrometry (CID-MS/MS) of ion mass-to-charge ratio (m/z) 1136.66, which allowed identification of the amino acid sequence MPLPPHPGHPG. (C) Mass spectrum of CID-MS/MS of ion m/z 1299.74, which allowed identification of the amino acid sequence LPPHPGHPGYIN. None of the five sequences was specific for amelogenin Y. Technical parameters of the spectral information obtained were: (A) 15 mV [sum = 990 mV] profiles 1–64 smooth Gauss 2 – baseline 10; (B) 98 mV [sum = 25181 mV] profiles 1–256 smooth Av 50 – baseline 150; and (C) 62 mV [sum = 15921 mV] profiles 1–256 smooth Av 50 – baseline 150.

protein sequences were also reported from Neanderthals, dating back ~75,000 yr, and samples were also obtained from bone (11).

Dental enamel has a greater resistance than bone to diagenesis (12), but so far complete proteins have not been extracted successfully from ancient teeth. This study tested the ability of a recently developed micro-etch technique (13) to collect adequate samples for processing for MS and for peptide fingerprinting, from both recently extracted and ancient teeth.

Material and methods

Enamel samples

Freshly extracted molars from different individuals were stored at −20°C until processing; on the day of the experiment, these molars were moved to room temperature until use (n = 2). We also used two ancient M3 teeth – one obtained from the Viking period (AD 800 to 1000) and the other from an early Christian period (AD 1000 to 1100) – from sites in Gotland, Sweden, for which conventional stable carbon

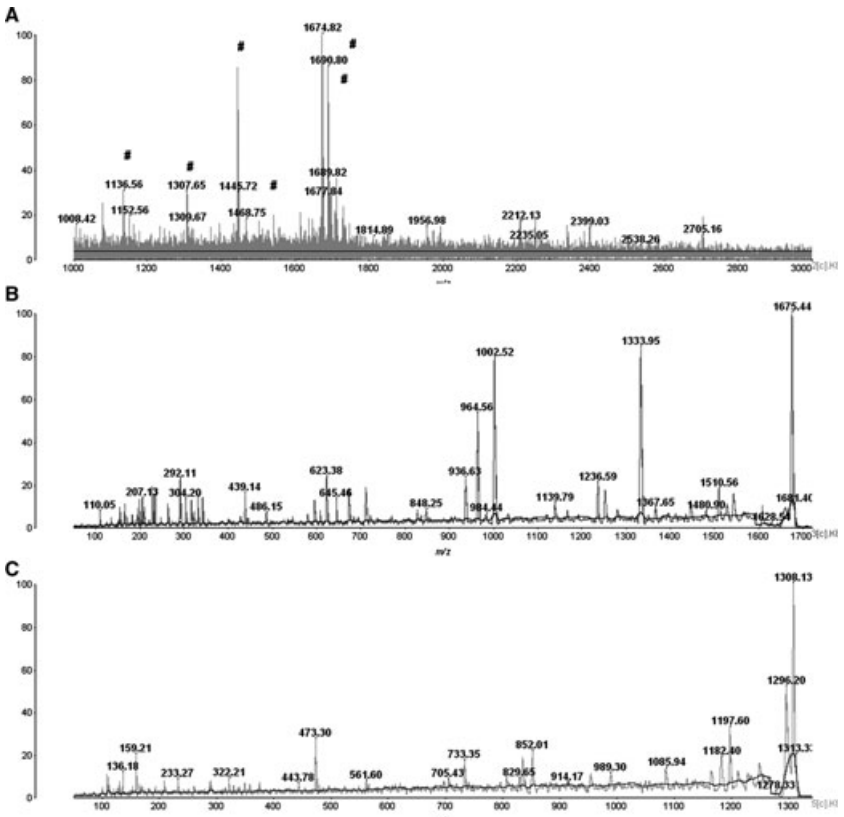

Fig. 3. (A) Mass spectrum of a superficial enamel extract of mummy teeth obtained by whole-crown etching in 10% HCl for 5 min. Six amelogenin X-isoform peptides were identified (protein score: 187). Ions identified as amelogenin. (B) Mass spectrum of collision-induced dissociation mass spectrometry/mass spectrometry (CID-MS/MS) of ion mass-to-charge ratio (m/z) 1674.82, which allowed identification of the amino acid sequence MPLPPHPGHPGYINF. (C) Mass spectrum of CID-MS/MS of ion m/z 1307.65, which allowed identification of the amino acid sequence WYQSIRPPYP. Technical parameters of the spectral information obtained were: (A) 16 mV [sum = 1022 mV] profiles 1–64 smooth Gauss 2 – baseline 10; (B) 32 mV [sum = 8238 mV] profiles 1–256 smooth Av 50 – baseline 150; and (C) 17 mV [sum = 4391 mV] profiles 1–256 smooth Av 50 – baseline 150.

(C) and oxygen (O) isotope analyses had already been conducted using well-established methods (14, 15). In this study the molars were washed with distilled water to clean the tooth surface.

This study was approved by the Institutional Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). Third molars were extracted from patients 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 including a tooth-donation term. Teeth were stored at −20°C until processing.

The use of protease inhibitors throughout this study was essential to avoid proteolysis. The inhibitors employed here were phenylsulfonylfluoride, *N*-ethylmaleimide, and phenanthroline, all of which were purchased from Sigma-Aldrich (St Louis, MO, USA) as powders. The protease inhibitors were prepared as stock solutions in methanol and diluted in buffer to the appropriate concentration (2 mM for all) just prior to use.

Protein-extraction technique (whole-crown etching)

The protein-extraction technique used here has recently been reported (13). The whole tooth crown was submerged for 5 min in 1 ml of 10% HCl containing proteinase inhibitors. The solution was transferred to a microcentrifuge filter of 30 kDa nominal cut-off (Ultrafree-MC microcentrifuge filters, M0536; Sigma-Aldrich) and centrifuged (30 min, 3,500 *g*, 4°C). The solution and proteins smaller than 30 kDa that passed through the filter were collected in the 'flow-through'. The flow-through was passed through a microtip filled with Poros 50 R2 resin (PerSeptive Biosystems, Framingham, MA, USA) to remove acid and salts, and the proteins bound to the porous resin were eluted with 50% acetonitrile/0.2% formic acid, and dried in a Speed Vac (AS 290; Savant, Rochester, NY, USA) for 30 min.

Digestion of proteins

Reduction/alkylation of proteins was performed as follows. Dried protein was suspended in 20 μl of 50% acetonitrile/100 mM ammonium bicarbonate, 5 μl of 45 mM dithiothreitol (DTT) was added, and the solution was incubated for 1 h at 56°C. Then, 5 μl of 100 mM iodoacetamide was added for 1 h in the dark, at room temperature. The samples were diluted five fold with 100 mM ammonium bicarbonate, 0.5 μg of trypsin was added, and trypsin hydrolysis was carried out for 22 h at 37°C. Finally, 5 μl of formic acid was added to quench the

reaction. The supernatants were desalted using a microtip filled with Poros 50 R2 resin, according to the procedure described above.

Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight

The samples were redissolved in a 5-mg ml^{-1} alfa-cyano-4-hydroxicinnamic acid (CHCA; Sigma-Aldrich) matrix with 50% acetonitrile/0.1% trifluoroacetic acid (TFA), crystallized in a matrix-assisted laser desorption/ionization (MALDI) target, and MS was accomplished using a MALDI time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (Axima Performance; Kratos-Shimadzu Biotech, Manchester, UK). External calibration of the mass spectrometer was performed using synthetic peptides (ProteoMass Peptide and Protein Maldi-MS Calibration Kit; Sigma-Aldrich). The mass accuracy was < 50 ppm. Peptides were analyzed for mass precursor and ion precursor selection by data-dependent acquisition following high-energy collision-induced dissociation (CID) to produce product ions of each precursor ion, which were then used for amino acid sequence determination.

The CID-MS/MS spectra were analyzed with the MASCOT software, version 2.2.04 (Matrixscience, London, UK), using the database Swiss-Prot (FASTA file 2011x; with 528048 sequences; 186939477 residues), with *Mammalia* or *Homo sapiens* used in the taxonomy, carbamidomethyl cystein as fixed modification, and methionine oxidation as variable modification. Peptide mass tolerance was $\pm$ 1.2 Da and fragment mass tolerance was $\pm$ 0.8 Da.

Results

Our results indicate that the superficial enamel etching technique tested here is very effective in generating adequate samples for MS analyses.

The MS graph in Fig. 1 shows the direct application of purified superficial enamel samples from mature human enamel without separation of samples in a gel. The results demonstrate that it is possible to obtain enough protein material of < 30 kDa for use in MS and MS/MS, rendering very good enamel protein peptide signals.

Figure 2A shows the spectrum of the sample obtained from a dry human (contemporary) molar. From this sample, five amelogenin peptides, all from the amino-terminal sequence, were identified. Although the MASCOT search rendered a higher score for amelogenin Y, all the sequences identified were common to both Y and X isoforms. Figure 2B shows the mass spectrum of the CID-MS/MS of ion mass-to-charge ratio (m/z) 1136.66, which allowed identification of the amino acid sequence MPLPPHPGHPG. Figure 2C shows the mass spectrum of the CID-MS/MS of ion m/z 1299.74, which allowed identification of the amino acid sequence LPPHPGHPGYIN.

Figure 3A shows the spectrum of a mummy molar from which amelogenin peptides were found. Figure 3B,C show amino acid sequences of two of these peptides; of particular interest may be the fact that the sequence WYQSIRPPYP is specific for the amelogenin X-isoform.

Discussion

The approach used in this study has many advantages over a previous study, carried out by others, in which attempts were made to characterize mature enamel using MS. In our study we characterized a small number of amelogenin peptides, mostly from the amino-terminus, and none was specific for the Y-isoform. One of the peptides characterized in the extract obtained from the mummy tooth was specific for the X-isoform of amelogenin. A previous study (16) identified an amelogenin peptide by comparison of the MS signal obtained with that of a synthetic peptide as the signal intensity was not high enough to allow the amino acid sequence to be identified directly from the MS data. Our samples gave MS peaks of sufficient resolution for direct identification of amino acids, in agreement with a recent study by our group using the micro-etching technique in contemporary mature human enamel (13). Furthermore, the technique used in this study is more conservative, preserves the dental crown, and avoids contamination with collagen from dentine, which occurred in the previous study (16).

Studies on the enamel of continuously growing teeth may also benefit from the recovery of proteins from the enamel of both contemporary (17) and past (18) species. In this regard, it is interesting that the ratios of C isotopes, formed at different times, clearly preserves seasonal changes in diet and local environmental conditions, and such restricted area-techniques may facilitate sampling.

Enamel also contains some proteins that remain after maturation [for instance, tuftelin and enamelin fragments; (19)]. Some enamel proteins are tightly bound to the enamel crystals (20), and hence worthy of further study by protein analysis to establish their preservation state in tooth remnants. This is particularly relevant because of the amazing protein data obtained from osteocalcin (10).

Another interesting feature of enamel is the fact that it is produced over many years if the whole dentition is considered; therefore, isotopic signatures found in enamel formed at different times have the potential to unravel information on some components of the diet since birth up to the age of $\sim$11 yr (in contemporary humans).

An amazing possibility in the study of past species is the use of data directly from tissue samples processed for stable-isotope MS, which can be accomplished using enamel (21, 22). The C and O isotopic signatures found in dental enamel may be complementary to those found in bone, aiding determination of important aspects of the diets of animals and hominins (23). While such isotopic signatures of enamel apatites can be reliably followed up in 2- to 3-million-yr-old hominin fossils, bone-apatite isotopes can only be reliably followed up in 200,000-yr-old fossils (23). While stable ratios of C isotopes provide information on the vegetation consumed (21), stable ratios of O isotopes in mammalian tissues inform on environmental conditions (24). Stable N isotopes provide information on the proteins in the diet (24) and may shed

light on aspects such as breastfeeding. So far, only C and O isotopes have been determined in enamel remains (25), with all the information on N isotopes having been derived from bone and dentin (24, 25). Therefore, the ability to recover protein from enamel could enhance the quality of the data on protein in the diet, as (theoretically) the enamel can harbor preserved protein for a much longer period of time.

The potential recovery of well-preserved protein fragments from the enamel may open new perspectives for the use of a tissue that contains preserved chemical and time information in its very structure. Protein has not yet been adequately searched for in enamel.

The new techniques described in our study may also be important for sex determination in forensic science because different isoforms of amelogenin are encoded by the X and Y chromosomes, which is the basis for its popularity in sex determination using PCR analyses (26).

The prospect of obtaining enamel samples from different layers of enamel may also facilitate analysis of microelements in archeological remains. Ratios between strontium isotopes have already been determined in enamel samples (27).

In conclusion, the recently described (13) micro-etch procedure is very effective in providing enamel samples that are adequate for protein analysis from dry teeth and ancient teeth; the amount of enamel required is much lower than for conventional techniques, and therefore this procedure is of potential in forensic and/or archaeological science studies.

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Conflicts of interest – The authors declare no conflicts of interest.

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