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Comprehensive proteomic analysis of human dentin

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Proteomic analysis of the human body is a significant recent scientific endeavour. In this study, we investigated the proteomic profile of human dentin using modern analytical and mass spectrometric techniques. Five healthy permanent human molars from five adults were cut, pulverized, denaturated with guanidine buffer, and demineralized with EDTA buffer. The extracted proteins were analysed by gel electrophoresis (SDS-PAGE and two-dimensional gel electrophoresis), digested with trypsin, and separated by liquid chromatography/high-resolution tandem mass spectrometry. We identified 289 proteins with high confidence, 90 of which had not been previously detected in human dentin. Nine (currently hypothetical) proteins were identified for the first time in an actual human sample. The proteins have a variety of functions, including calcium-ion binding, formation of the extracellular matrix, formation of the cytoskeleton, cytoskeletal protein binding, immune response, and transport. In conclusion, this is the first use of two-dimensional electrophoresis for investigating human dentin.

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Proteomics of different types of human cells, organs, tissues, and fluids has been a major research topic in life sciences worldwide for the last few decades, and useful basic data for a variety of target tissues are still greatly needed (1).

The human tooth is the hardest part of the human body and it has a specific structure and composition. Its main mineralized parts are enamel, dentin, and cementum. Another tooth tissue is the pulp, which contains blood vessels and nerve fibres. Dentin is a highly mineralized tissue that forms the bulk of the tooth, serving as a protective covering for the pulp and providing support for the overlying enamel and cementum. The tooth also contains predentin, which is unmineralized. Predentin is located between the odontoblasts and the mineralized dentin and is present for the lifespan of the tooth. The dentinal tubules, which run between the pulp and the dentin, are where the odontoblasts operate (2).

Odontoblasts are the key cells responsible for the formation of dentin. They are highly specialized cells and are closely associated with the formation and maintenance of dentin. They produce and secrete the organic components of the extracellular dentin matrix (2). Evidence exists to show that the dentin extracellular protein matrix is formed first of all and then dentin mineralization is initiated on the basis of the protein deposited (3).

On a weight basis, mature dentin is approximately 70% mineral, 20% organic matrix, and 10% water. To date, the organic matrix of dentin has been reported to contain a predominantly collagenous matrix that consists of approximately 85–90% of the total protein (4). In humans, there are at least 27 known types of collagen, expressed from 42 different collagen genes (5). Collagen type I is the most abundant collagenous protein in the dentin matrix, which also contains smaller amounts of type III, V, VI, XI, and XII collagens (6, 7). Collagen fibrils provide a three-dimensional scaffold for the deposition of apatite crystals (8).

The most common tooth disease is dental caries, which is present in >90% of all adults. Hereditary dentin disorders are divided into five types: three types of dentinogenesis imperfecta; and two types of dentin dysplasia (9). They are characterized by inherited dentin defects that not only affect collagen but also cause other protein disorders (10, 11).

The presence of collagen in dentin correlates closely with the presence of tissue matrix metalloproteinases (MMPs) 2, 9, and 20 (12, 13). Fibronectin has also been found in association with collagen fibrils in the predentin (6).

The major noncollagenous protein in human dentin is dentin sialophosphoprotein (DSPP). This protein is critical for proper dentin mineralization because genetic defects in DSPP cause dentin dysplasia type II and dentinogenesis imperfecta types II and III (14). During tooth formation, DSPP is expressed by odontoblasts and pre-ameloblasts, and its translation products accumulate in the dentin matrix. Dentin sialophosphoprotein is rapidly cleaved into three products: dentin sialoprotein, dentin glycoprotein, and dentin phosphoprotein (15, 16). It is believed that these proteins play

regulatory roles in the nucleation of hydroxyapatite onto dentin matrix collagen and in the subsequent growth of the hydroxyapatite crystals (4, 17). It is assumed that dentin sialoprotein is modified by carbohydrates and this leads to unusual behaviour on SDS-PAGE (16).

Sialic acid-rich proteins are further constituents of the non-collagenous proteins present in dentin. Besides dentin sialoprotein, this category includes osteopontin, osteonectin, osteocalcin, bone sialoprotein, bone acidic glycoprotein, dentin matrix protein I, and integrin-binding sialoprotein (18). These sialic acid-rich proteins share some common features, such as the presence of relatively large amounts of sialic acid, and phosphate groups. These proteins also play an important role in the highly sophisticated process of dentin mineralization (17).

Dentin matrix protein I is a multifunctional acidic protein involved not only in the biomineralization of dentin and bones, but also in phosphate homeostasis and in the differentiation of odontoblasts and osteoblasts (19). Dentin matrix protein I contains a large number of phosphorylated serines. In the presence of calcium it undergoes self-assembly into filaments. Dentin matrix protein I specifically binds to the N-telopeptide sequence of collagen and has been shown to affect collagen fibrogenesis. Periostin (20), osteocalcin, and osteonectin (21) also have distinct roles in the dentin mineralization process.

Furthermore, a number of proteoglycans, which belong to the family of small leucine-rich proteoglycans, have been identified in predentin and dentin (22), and two family members (biglycan and decorin) bind to collagen fibrils and hydroxyapatite crystals (23). Lumican, fibromodulin, and osteoadherin are also involved in the odontogenesis processes (2).

In recent studies, the proteome of dentin has been reported to be a complex mixture of proteins with various functions (e.g. in metabolism, cellular organization, signal transduction, cell growth, homeostasis, the regulation of biological processes, stress response, transport, immune response, transcription factor activity, and nucleic-acid binding) as well as proteins with unknown functions (6, 24).

As the proteomic analysis data for human dentin are incomplete, this study was carried out to extend the proteome list of human dentin using a comprehensive analytical tool: two-dimensional gel electrophoresis (2D-GE) followed by nano liquid chromatography tandem mass spectrometry (nLC-MS/MS). We believed that the results would provide a clearer understanding and better characterization of the biological processes that take place in human dentin.

Material and methods

Sample preparation

Five healthy erupted permanent human third molars were extracted for clinical reasons from five adults (two women and three men). The teeth were taken from patients, 22–23 yr of age, in a dental clinic after acquiring their informed consent for tooth donation for research.

The teeth were washed thoroughly with water, the cementum and soft tissues were mechanically scraped off with an iron spatula, and the teeth were cleaned with brushes. Every tooth was then cut horizontally (below the level of the enamel) and only the subjacent part (the roots) was used for the experiment. The roots were crushed in a jaw vice into smaller fragments, and the dental pulp was carefully removed. These smaller fragments of dentin were frozen in liquid nitrogen and pulverized to a powder with a mortar and pestle. The dentin samples taken from the roots were about 1.8 g wet weight (all five teeth pooled).

The prepared dentin samples were sequentially extracted, first of all with guanidine buffer and then with disodium ethylenediaminetetraacetic acid (EDTA) buffer, according to the modified protocol (25) shown in Fig. 1. Protease Inhibitor Cocktail Tablets (Roche Applied Science, Mannheim, Germany) were included in each buffer during the extraction procedures. In brief, the dentin powder (1.0 g) was suspended in 2.5 ml of a guanidine buffer (6 M guanidine, 1.2 M Tris–HCl, 2.5 mM EDTA, pH 8.4) for 72 h at 4°C. After centrifugation (1,000 ***g***, 10 min), the guanidine supernatant was removed and the pellet was washed with 0.5 ml of water and recentrifuged (1,000 ***g***, 10 min, 4°C). The aqueous supernatant was pooled with the guanidine supernatant to obtain the 'G-extract'. The G-extract was subsequently reduced by the addition of 100 μl of 1 M dithiothreitol and heated at 37°C for 1 h with shaking. *S*-carbamidomethylation (alkylation) was then carried out by incubation with 500 μl of 1 M iodoacetamide for 30 min at ambient temperature in the dark. Alkylation was stopped by the addition of 100 μl of 1 M dithriothreitol. The samples were finally desalted either by dialysis or with Econo-Pac 10DG desalting columns (Bio-Rad, Hercules, CA, USA).

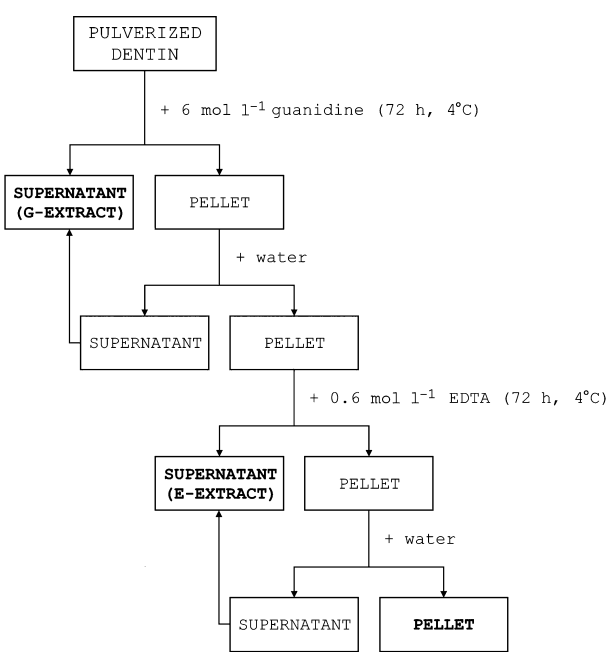

Fig. 1. Flow chart of the sequential extraction procedures used to prepare human dentin protein for analysis by gel electrophoresis. Samples given in bold were analysed.

For advanced sample treatment, the pellet was suspended and demineralized in 5 ml of 0.6 M EDTA buffer (pH 8.0) for 72 h at 4°C. After centrifugation (1,000 ***g***, 10 min, 4°C), the EDTA supernatant was removed and the pellet was washed with 0.5 ml of water and recentrifuged (1,000 ***g***, 10 min, 4°C). The aqueous supernatant was pooled with the EDTA supernatant to obtain the 'E-extract'. The remaining pellet was washed with 500 μl of water, then this second wash of pellet was discarded and the pellet was resuspended in 5 ml of water. The E-extract and the pellet were subjected to the same reduction/alkylation procedure as described for the G-extract in the previous paragraph. All samples were finally dialysed, lyophilized, and stored frozen at −80°C.

The lyophilized samples (G-extract and E-extract) were treated with Amicon Ultra-0.5 centrifugal filter devices (Millipore, Billerica, MA, USA), which have a nominal molecular mass cut-off of 100 kDa, according to the manufacturer's instructions. Briefly, the samples were dissolved in 0.5 ml of 0.1% formic acid at a concentration of 2–6 mg ml^{-1} and centrifuged at 14,000 ***g*** and 4°C for 30 min. The filtrate was collected, evaporated to dryness, and analysed by 2D-GE.

The protein concentration was determined with the Bradford assay using the Bradford Dye Reagent and serum albumin standard (Bio-Rad).

Separation by SDS-PAGE

One-dimensional polyacrylamide gel electrophoresis (SDS-PAGE), using a homogeneous 12.5% polyacrylamide resolving gel of 1 mm thickness, was performed essentially following the methods of Laemmli (26). Gels were run in a Mini-Protean Tetra cell system (Bio-Rad) at 200 V until the Bromophenol Blue dye reached the end of the gel (approximately 45 min). The gels were then stained with Bio-Safe Coomassie Blue G250 stain (Bio-Rad). After staining, the gels were washed with water, scanned with a GS-800 Calibrated Densitometer, and processed using image analysis software (Quantity One; Bio-Rad). The lanes with protein samples were cut into 10 pieces of equal size and processed using the in-gel digestion procedure.

Separation by 2D-GE

Part of the lyophilized sample (0.3 mg or eventually 1.0 mg) of protein was solubilized in 130 μl of rehydration solution (7 M urea, 2 M thiourea, 2% (w/v) 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulphonate (CHAPS), 0.2% (w/v) Bio-Lyte ampholytes (3–10 buffer), and 1% (w/v) dithiothreitol). Proteins were then transferred to Ready Strip IPG Strips (pH 3–10 non-linear, 7 cm; Bio-Rad) overnight by active in-gel rehydration (50 V, 20°C).

Isoelectric focusing was carried out at 20°C with a Protean IEF cell system (Bio-Rad) under mineral oil. Proteins were focused in four steps: 250 V for 20 min, linear gradient; 500 V for 1 h, linear gradient; 1,000 V for 1 h, linear gradient; and 4,000 V for 20,000 Vh (Volt-hours), rapid gradient. Before separation in the second dimension, the strips were equilibrated according to Görg *et al.* (27). After the equilibration step, the strips were rinsed in Tris–glycine buffer (pH 8.3), transferred to a homogeneous 12.5% SDS-polyacrylamide gel (with the same composition as in the previous section), and overlaid with 0.5% (w/v) Certified Low Melt Agarose (Bio-Rad) in SDS-PAGE running buffer containing a trace of Bromophenol Blue. Finally, 5 μl of Precision Plus Protein Standards (molecular mass range 10–250 kDa; Bio-Rad) was added to the top end of the gel.

Gels were run in the Mini-Protean Tetra Cell system and were further processed (i.e. by staining, scanning the gel, and cutting the spots) as described for SDS-PAGE in the previous section. SDS-PAGE and 2D-GE gel analyses were repeated at least once.

In-gel digestion

Protein spots (approximately 1–2 mm in diameter) from 2D-GE or protein bands from SDS-PAGE were excised from the Coomassie Blue-stained gels and then processed as described by Shevchenko *et al.* (28). Before the in-gel digestion, the gel pieces were cooled in an ice-cold bath and swollen in 50 μl of digestion buffer (50 mM ammonium bicarbonate) containing trypsin (30 μg ml^{-1}, type IX-S; Sigma, St Louis, MO, USA). After 1 h of cooling at 4°C, the gel pieces were placed in an incubator with thermostatted air circulation and incubated overnight at 37°C. The volumes of solutions needed to process the protein bands were approximately fourfold larger than the volumes needed to process the spots.

The resulting tryptic peptides were extracted from the gel pieces by the addition of 150 μl of extraction buffer (5% formic acid and 30% acetonitrile in water) and sonication for 15 min. The buffer was then replaced and the gel pieces were sonicated again for a further 15 min. After each extraction step the solutions were spun and the supernatants were removed, pooled, and concentrated to dryness in a vacuum centrifuge. Dried extracts were stored at −80°C before analysis.

Analysis of tryptic digests with LC-MS/MS

Dried protein digests were dissolved in 20 μl of 0.1% formic acid, centrifuged, and the supernatant was transferred to inserts in vials.

The nano-high-performance liquid chromatography (HPLC) apparatus used for analysing the protein digests was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a maXis quadrupole time-of-flight (Q-TOF) mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany) by an nanoelectrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 (Bruker Daltonics) and micrOTOF-control 3.0 (Bruker Daltonics). The data were collected and manipulated with the software packages ProteinScape 2.0 and DataAnalysis 4.0 (Bruker Daltonics).

Five microliters of the peptide mixture was injected into an NS-AC-11-C18 Biosphere C18 column (particle size, 5 μm; pore size, 12 nm; length, 150 mm; and inner diameter, 75 μm), with an NS-MP-10 Biosphere C18 precolumn (particle size, 5 μm; pore size, 12 nm; length, 20 mm; inner diameter, 100 μm), both manufactured by NanoSeparations (Nieuwkoop, the Netherlands).

The separation of peptides was achieved via a linear gradient between mobile phases A (water) and B (acetonitrile), both containing 0.1% (v/v) formic acid. Separation was started by running the system with 5% mobile phase B, followed by gradient elution to 30% B at 70 min. The next step was a gradient elution to 50% B in 10 min, and then a gradient to 100% B in 8 min was used. Finally, the column was eluted with 100% B for 2 min. Equlibration before the next run was achieved by washing the column with 5% mobile

phase B for 10 min. The flow rate was 0.25 μl min^{-1}, and the column was held at ambient temperature (25°C).

Online nano-electrospray ionization (easy nano-ESI) in positive mode was used. The ESI voltage was set to +4.5 kV and the scan time to 1.3 Hz. The operating conditions were: drying gas (N_2), 1 l min^{-1}; drying gas temperature, 160°C; and nebulizer pressure, 0.4 bar. The experiments were performed by scanning from 100 to 2200 m/z. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (m/z = 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling accurate determination of molecular mass. All LC-MS/MS analyses were performed in duplicate.

Database searching

Data were processed using ProteinScape software (Bruker Daltonics). Proteins were identified by correlating tandem mass spectra to the IPI and SwissProt databases, using the MASCOT search engine (http://www.matrixscience.com). The taxonomy was restricted to *Homo sapiens* to remove protein identification redundancy. Trypsin was chosen as the enzyme parameter. One missed cleavage was allowed, and an initial peptide mass tolerance of ±10.0 p.p.m was used for MS analysis and of ±0.05 Da for MS/MS analysis. Cysteines were assumed to be carbamidomethylated, proline and lysine to be hydroxylated, and serine, threonine and tyrosine to be phosphorylated; methionine was allowed to be oxidated. All these possible modifications were set to be variable. The monoisotopic peptide charge was set to 1+, 2+, and 3+. The Peptide Decoy option was selected during the data-search process to remove false-positive results. Only significant hits (a MASCOT score of ≥ 60; http://www.matrixscience.com) were accepted.

Results

Five, third-molar teeth were pooled to obtain a sufficient amount of sample. Great care was taken to remove any remnants of soft tissue, enamel, and tooth pulp within the dentin-sample preparation procedure. The whole procedure for protein extraction from dentin (Fig. 1) was based on two sequential extractions of the pulverized dentin sample – the first with guanidine buffer and the second with EDTA buffer. Thus, three partial samples (G-extract, E-extract, and pellet) were obtained from the primary dentin sample.

Two-dimensional electrophoresis was employed to identify more proteins than in previous studies (6, 24). Use of a small format of 2D-GE (7 cm) enabled us to analyse relatively small amounts of sample. IPG strips with a broad pH range (pH 3–10) were used with the aim to cover as many proteins as possible in the sample. Samples were analysed by 2D-GE and SDS-PAGE: analyses of G-extract, E-extract, and pellet are shown in Fig. 2.

Besides 2D-GE, SDS-PAGE was primarily used for the first approximation step and to verify proper desalting of the extract and pellet samples. The proteins in the SDS-PAGE gels were also analysed. The whole gel lanes with protein samples were cut into 10 pieces of equal size and then subjected to the in-gel digestion procedure and nLC-MS/MS analysis. In total, 59 unique proteins were detected in the SDS-PAGE gels of the G-extract, E-extract, and pellet (Tables S1 and S2). Almost all of these proteins were detected again in the 2D-GE gels, except for one protein, COL2A1 (the definitions of protein abbreviations are given in Table S1).

Identification of the proteins and peptides was based on searches of the IPI and SwissProt databases. The data sets of dentin proteins were arrayed by excluding the overlapping proteins and removing false-positive results. Using this approach, in total 289 proteins from the dentin matrix were identified in the 2D-GE gels of G-extract and E-extract, together with the SDS-PAGE gel of the pellet. These proteins were separated into two groups and are shown in Tables S1 and S2. Table S1 shows 269 proteins that form the main part of the protein matrix in dentin. The proteins were arranged by their MASCOT score and by the number of

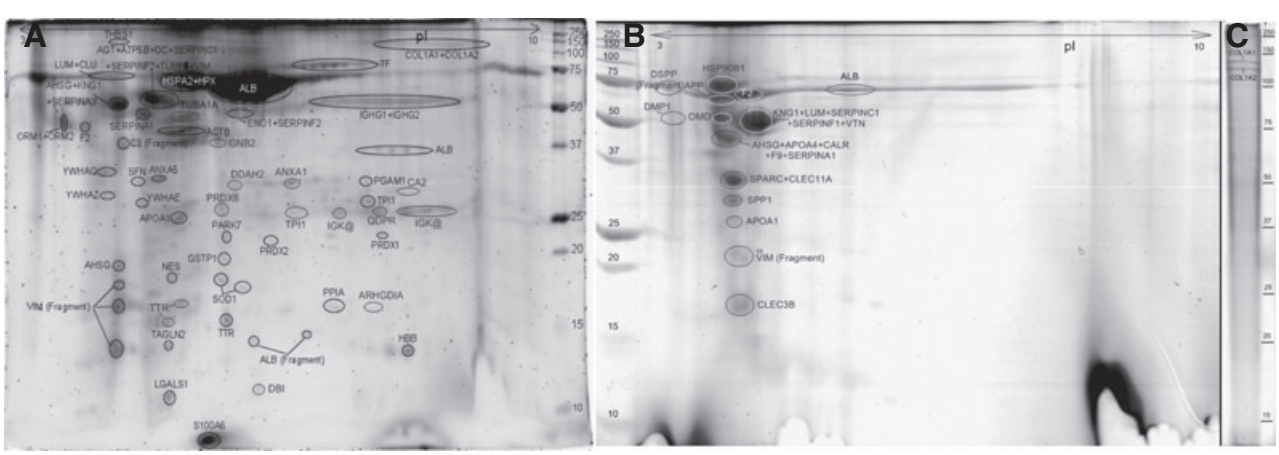

Fig. 2. Two-dimensional gel electrophoresis (2D-GE) images of 1.0 mg of G-extract (A) and 0.3 mg of E-extract (B) and an SDS-PAGE gel image of 0.3 mg of pellet (C) from healthy human dentin tissue. All samples were separated by electrophoresis through 12.5% (%T, which is the weight percentage of the total acrylamide monomer including the bis-acrylamide crosslinker) acrylamide/bisacrylamide gels, and the gels were stained with Coomassie Brilliant Blue dye. IPG strips (pH 3–10, non-linear) were used in the 2D-GE. The definitions for the symbols used to label the spots are given in Table S1.

peptides that identified the proteins. Some proteins were identified in multiple spots, indicating the presence of protein isoforms and fragments. This was especially the case for albumin, collagens, and vimentin.

The remaining 20 proteins found in dentin, perhaps skin and laboratory dust contaminants, are shown in Table S2. These proteins are almost all keratins. They could either be an integral part of the protein matrix in dentin, but can be more plausibly attributed to contamination of the samples with skin (29). Some of these proteins were present in some of the spots throughout the 2D-GE gel; moreover, they were present in the sample buffer-only control lane.

As shown in Table S1, the most abundant protein type in the dentin matrix is collagen. We found the two most abundant collagens to be COL1A1 and COL1A2. The other 'top ten' most abundant proteins in dentin were: ALB, AHSG, F2, VIM, SERPINF1, SERPINA1, SERPINC1, and OMD (ordered according to their score and unique peptide hit number). This is in good approximate agreement with previous observations on dentin tissue (6, 24). In addition to these proteins, several non-collagenous proteins were identified, including BGN, PCOLCE, LUM, DSP, SPARC, MMP20, DSPP, GSN, CHAD, HSPG2, DMP1, and DCN (Table S1). The enamel proteins ANXA5, ANXA1, and ANXA2 were also identified. Proteins related to odontogenesis, extracellular matrix function, and mineralization were found. They included THBS1, FMOD, MGP, TIMP1, and ECM2. We also identified several bioactive molecules, such as IGFBP5, POSTN, TNC, JUP, and IGFBP1. Collagen-degrading proteins (MMP20 and CTSD), which are involved in dentine modification with aging, were also identified. In addition, we detected some minor collagens (COL2A1, COL3A1, COL5A1, COL5A2, COL11A2, and COL12A1) in the dentin matrix. This supports the observation, of a previous report, which found pro-COL5A1, pro-COL5A2, pro-COL11A1, and pro-COL11A2 chains in mouse incisor teeth (30).

Interestingly, we identified nine currently hypothetical or putative uncharacterized proteins with the following IPI numbers: IPI00384938, IPI00399007, IPI00402710, IPI00418931, IPI00423466, IPI00426051, IPI00449920, IPI00550731, and IPI00784950 (marked with a § symbol in Table S1). Our measurements are the first evidence for the existence of these proteins in actual human tissue.

Discussion

Proteomic research at present mainly includes the establishment of 2D-GE reference maps and a database of organs, tissues, and cells. This study is the first to use 2D-GE for the proteomic investigation of dentin from human teeth.

Dentin sample preparation and 2D-GE analysis

The two-step sequential extraction technique (guanidine followed by EDTA) was used with great success in 1980 for the study of non-collagenous proteins in rat incisor dentin (31). Enzyme inhibitors were included in the extraction buffers to avoid unintentional degradation and loss of some dentin proteins. In the more recent complex proteomics study of human dentin, samples were extracted for 15 d with EDTA, pulverized, then separated by SDS-PAGE (6). Methods for extracting proteins from the dentin matrix using EDTA solution have also been described (24, 25). These experiments demonstrated that some matrix proteins (e.g. osteocalcin, calreticulin, DSPP, extracellular matrix proteins, MMPs, and collagens) are soluble either in guanidine buffer or in EDTA solution. Thus, by adapting all of the previous approaches, we used two-step sequential extractions of the pulverized sample, with higher concentrations of guanidine and EDTA in the extraction and demineralization buffers, to release as many proteins as possible (Fig. 1). By combining the denaturation and demineralization steps, more proteins were obtained from the calcific sample compared with use of a one-step extraction procedure. We conclude that proteins are released more effectively as a result of combining these dentin scaffold destruction steps. Prolonging the period of pellet extraction (above 72 h) or further repetitions of the extraction and demineralization steps did not increase the number of proteins found.

SDS-PAGE was used as a reference method for 2D-GE. Using this method, we were able to identify 59 valid proteins in the dentin samples (G-extract, E-extract, and pellet), far fewer than was found in the 2D-GE gels. Despite the higher separation power of 2D-GE, the protein COL2A1 was found only in SDS-PAGE gels. This could be a result of some limitations of the 2D-GE method, such as difficult solubilization of hydrophobic proteins that cannot penetrate the IPG strips. Additionally, boiling the samples with SDS solution during sample preparation could enhance the release of collagen fibrils from the dentin matrix, thus making it possible to analyse and identify them by SDS-PAGE.

A standard procedure of Görg *et al.* (27) was used for 2D-GE analysis of the dentin samples. The dentin samples have a relatively high collagenous protein content. Type I collagens form by far the majority of the dentine protein mass, a fact that makes 2D-GE analysis of the dentin sample somewhat difficult. As seen in Fig. 2, a vertical streak was found in the basic pH region (pH ~9) of all of the 2D-GE analyses of the G-extract (Fig. 2A) and the E-extract (Fig. 2B). These streaks were formed mainly of collagens. Also, intensive and large spots of albumin and its isoforms were found in these gels. These high-abundance proteins can reduce the resolving power of 2D-GE so that many low-abundance proteins become undetectable. In an attempt to circumvent this problem, we used an ultrafiltration method with a cut-off limit of 100 kDa to reduce the content of high-molecular-weight collagen, but without success. This was probably because of the many collagen fragments of variable length that were also present in the sample.

Only approximately 60 valid proteins were identified in the 2D-GE gel when 0.3 mg of the G-extract was analysed. Therefore, to investigate more proteins, especially the low-abundance proteins, a higher load of the G-extract (1.0 mg) was applied to the IPG strip (Fig. 2A). More protein spots were revealed, especially in the area below ~60 kDa, enabling their detection and identification. On the downside, the sample load led to the partial precipitation of proteins, mainly collagens, and the observation of 'smearing' of bands along the direction of flow. The possible aggregation of proteins into larger clusters added to this problem and reduced the resolution in the area from 60 to 250 kDa. Our 2D-GE analyses were carried out using broad-pH-range IPG strips (pH 3–10, non-linear) to ensure that the investigation was of the whole proteome of dentin tissues.

The separation and identification power of 2D-GE was better with the G-extract samples than with the samples of E-extract, probably because of the nature of the target proteins. We observed some relatively large and unclear spots of many unresolved proteins in the acidic pH region of the E-extract (Fig. 2B). These spots remained repeatedly unresolved, even when much smaller amounts of E-extract (<0.1 mg) were loaded onto the strip. A representative image of the E-extract was obtained when 0.3 mg of the sample was separated by 2D-GE (Fig. 2B). Naturally, more proteins were revealed when 1.0 mg of the E-extract was analysed. Many dentin matrix proteins (e.g. phosphoproteins) are considered to be highly acidic because of their phospho groups. The limitations of the accessible pH range of the IPG strips used (between pH 3 and pH 10) make the isoelectric focus analysis of very acidic proteins almost impossible. Moreover, some dentin proteins can be glycosylated. This leads to unusual behaviour of these proteins during gel electrophoresis (15).

As expected, analysis of the pellet sample revealed only 15 valid proteins. Moreover, collagens were by far the most abundant proteins in the pellet (Fig. 2C) and therefore the 2D-GE analysis of the pellet did not produce a useful gel.

The 2D-GE method is able to detect post-translational modifications and isoforms of proteins. Several spots corresponding to the same protein were detected (e.g. serum albumin, apolipoprotein A-I, and serotransferrin). A large number of spots corresponded to vimentin. The majority of these spots represented fragments of the precursor protein.

Identified dentin proteins

To date, two complex proteomic studies investigating human dentin proteins have been carried out, using one-dimensional electrophoresis followed by MS/MS identification of the proteins (6, 24). SDS-PAGE revealed, in total, 233 proteins in three teeth in the earlier study. Similarly, 147 EDTA-soluble tooth proteins were identified in the more recent study. These findings are truly impressive and represent the most extensive list of human dentin proteins so far. However, neither study presented the score and the number of the unique peptides for each published protein. The relatively high number of identified proteins might be explained by the different methodological approaches used in these studies. In our study we applied a stringent validation procedure using the protein score as the major criterion, and thus the number of identified proteins in this study is not fully comparable with the number of proteins identified in the two previous studies.

As mentioned in the Results, we summarized the identified proteins in Tables S1 and S2. The proteins were ranged in descending order of their MASCOT score. This is not a fully quantitative criterion, because matches using mass values (either peptide masses or MS/MS fragment ion masses) are always handled on a probabilistic basis. The total MASCOT score is the absolute probability that the observed match is a random event. It is generally true that proteins which are measured and identified with a higher certainty always have a higher score. More information about the MASCOT score is available at http://www.matrixscience.com.

We used 2D-GE for the analysis of dentin samples because of its better separation power. Using this approach we confirmed the presence of 126 proteins also found in two previous studies (6, 24). Comparison of the proteins in Table S1 with previously mentioned articles revealed an 'additional' 143 proteins that were not previously detected in these studies of human dentin. The molecular functions of these proteins are very broad and include calcium ion binding (CALB1, CALM, CALML3, CALML5, NUCB2, S100A6, S100A7, and S100A9), cytoskeletal protein binding (DPYSL2, MAP1B, and TPM3), cell adhesion molecule activity (DSG1, DSC1, IGFBP1, JUP, and LGALS7), formation of the extracellular matrix (COL3A1, COL5A2, DMP1, MEPE, and SPOCK), formation of the cytoskeleton (DSP, MICALL1, MYL6, NEB, and TUBA1A), immune responses (IGHA2, IGHG1, IGHG3, ORM1, and ORM2), peptidase activity (CASP14, CTSD, and SCRN1), protease inhibitor activity (ITIH4, PEBP1, SERPINB3, SERPINB4, SERPINF2, and SERPING1), transporter activity (APOA2, HP, LTF, and SERPINA6), and unknown functions (ALPK2, ARHGDIA, CEP290, HYDIN, LRG1, LRRC4B, STYXL1, TMC2, TMEM198, and ZG16B). These findings demonstrate the ability of the methodology used in this work to investigate as many proteins as possible simultaneously.

Some of the 'additional' proteins have been previously detected in various parts of the human or animal body related to teeth. In human enamel or enamel epithelia, fibroblast growth factor receptor 1 and calpain were found, respectively (32, 33). Lactoferrin was detected by 2D-GE in *in vitro* pellicle-composition experiments (34). Ameloblasts produce calmodulin, a calcium-dependent modulator protein involved in the enamel mineralization process (35). Ameloblasts also produce histone H3 in tooth germs (36).

Odontoblasts produce tropomyosin, myosin, actin, and tubulin (37, 38). Extracellular matrix is predomi-

nantly expressed in osteoblasts and odontoblasts and plays key biological roles in bone and dentin metabolism (39). Dental papilla cells produce histone H4 (40), and dental follicle precursor cells form transgelin (41). Human pulp fibroblasts and odontoblast-like cells were shown to express desmoplakin and junction plakoglobin (42). Dental pulp cells produce various extracellular matrix components (e.g. osteocalcin) (43). Proteins S100A7, S100A8, and S100A9 are broad-spectrum antimicrobial peptides produced in the oral cavity by muconasal keratinocytes as constituents of the innate immune system (44). Interestingly, elevated levels of S100A8 and S100A9 were detected in pulpal tissue samples of carious teeth (45).

In periodontal ligament tissues, angiotensinogen and insulin-like growth factor-binding proteins 1 and 3 were studied (46, 47). Elevated expression of testican-3 was found in the periodontal ligament of mandibular third molars performing vertical movement compared with maxillary second premolars with occlusal contact (48). Higher levels of immunoglobulins (Ig heavy chain V-III region) were detected in the saliva of patients with periodontitis (49). The pellicle layer (an early defence barrier) of bovine teeth included carbonic anhydrase 1 (50).

We found a leucine-rich alpha-2-glycoprotein precursor in dentin. This is a serum glycoprotein of generally unknown function that has shown promise, based on qualitative assessments, as a biomarker for certain diseases, including microbial infections and cancer (51). Its constitution involves leucine-rich repeat protein domains. The role of leucine-rich alpha-2-glycoprotein in dentin remains unknown, but it can be anticipated, based on structural similarity, that its function might be similar to a large family of leucine-rich proteoglycans that organize the collagen network for the receipt of phosphoproteins and phospholipids.

We also detected the other predominant small leucine-rich proteoglycans, such as decorin, biglycan, lumican, mimecan, chondroadherin, and fibromodulin, in dentin. The rigid, spatially oriented glycosaminoglycan chains on decorin and biglycan are known to bind calcium and may feature directly in mineral initiation (22). Lumican has been immunolocalized in predentin and dentin, and its role in dentin may be in organization of the collagen network before mineral deposition (52). Fibromodulin also binds to fibrilar collagens and even competes with lumican for the same binding sites on collagen fibrils (53).

This study also confirms the existence of nucleobindin in dentin. Nucleobindin, a calcium ion-binding protein, was expressed in mature odontoblasts and in the dentin matrix, where it may contribute to the accumulation and transport of Ca^{2+} ions during mineralization (54). Tropomyosin-related kinases are expressed during tooth development (55). The temporal-spatial specific pattern and unique colocalization of dentin sialophosphoprotein and mimecan in mouse teeth suggest that they play complementary roles during odontogenesis (56).

We identified 269 proteins in the dentin, 90 of which (marked with a cross in Table S1) had not previously been detected in human dentin, but many of these 'novel' proteins had been detected in other human or animal tissues. There is not enough space here for a comprehensive discussion of all the 'novel' proteins in this study. Hence, only some were chosen for discussion in respect to their possible function in dentin.

We found a large number of proteins (immunoglobulins) and their variants participating in the immune response in the dentin. Immunoglobulins (e.g. IGH@ protein, Ig gamma-1 chain C region, Ig gamma-2 chain C region, Ig gamma-3 chain C region, and IGKC anti-RhD monoclonal T125 kappa light chain) can play a role in protecting dental tissues against plaque bacteria and in the development of dental caries. Some have even been detected in the human proteome for the first time.

Nebulin is a large filamentous protein that is an integral component of the skeletal muscle thin filament. Recent research in mice indicates that nebulin performs a wide range of functions. Besides the role of nebulin in the regulation of muscle contraction, it also plays a role in calcium homeostasis (57). We suggest that nebulin might have a similar function in dentin tissue.

Serine/threonine/tyrosine-interacting-like protein 1 is a catalytically inactive phosphatase that regulates mitochondrial-dependent apoptosis (58). Zymogen granule protein 16 homolog B has been recently shown to play a role in gene regulation and cancer metastasis (59).

The biomineralization of bovine bones was extensively studied. Among other proteins, serum albumin, alpha-2-HS glycoprotein, decorin, biglycan, osteonectin, collagen alpha-2(I) chain, secreted phosphoprotein 24, chondroadherin, lumican, thrombospondin-1, nucleobindin, osteomodulin, and histone H2B were shown to interact directly with calcium phosphate mineral (60). High expression of the *Hist1h2bb* gene was observed in mouse dental epithelia isolated from the initiation stage of tooth development (61). In our study we can confirm, for the first time, the presence of histone H2B type 1-B in human dentin.

Transmembrane channel-like protein 2 and teneurin-1 are two of the many transmembrane proteins that are expressed in developing nervous systems. Alpha-protein kinase 2 is a novel candidate gene for inherited hypertension in Dahl rats (62). Secernin 1, a cytosolic protein, appears to be involved in the regulation of exocytosis from mast cells (63). It might also be involved in the exocytosis of odontoblasts. Transmembrane protein 198 is a membrane scaffold protein that promotes LRP6 phosphorylation and Wnt signalling activation in human embryonic kidney cells (64). This protein might play an important role in the signalling pathways of odontoblasts. Despite the above speculations, it must be strongly emphasized that the exact functions of the 'novel' proteins in dentin remain unknown and are therefore worth investigating further.

One of the frequent problems encountered by investigators in proteomics is the cross-contamination of samples with skin keratins, other dermal proteins, or

proteins from laboratory dust. Regardless of working as carefully as possible and using laboratory glassware and chemicals that are as clean as possible, it is very difficult to completely avoid this contamination during processing of the sample. Thus, some traces of keratins and other dermal proteins are present in most samples analysed, and it is practically impossible to determine whether the keratins originate from the sample or from the contamination (29). Therefore, and despite the fact that the keratins were discussed with the other observed proteins in previous studies, we decided not to include the keratins and other possible dermal proteins and contaminants found in the dentin samples (20 proteins shown in Table S2) in the above discussion.

It is anticipated that the proteome of human dentin has still not been fully discovered. Owing to the limitations of the 2D-GE separation procedure, proteins with extreme pH values could not be separated. These proteins may be heavily modified (e.g. phosphorylated). Moreover, proteins with a molecular weight that was too low <10 kDa or too high >250 kDa could not be analysed. Another limitation of 2D-GE is its inability to detect proteins of very low abundance.

The proteins identified in this study were either constituents of the extracellular dentin matrix or could be associated with cellular functions and participate in the odontoblast processes taking place in dentinal tubules. Many intracellular proteins could be constituents of odontoblasts located on the inner surface of dentin, with their fibres pervading deep into dentinal tubules.

All of the human dentin proteins identified in this study have a variety of functions. Their functions in biological processes were categorized according to the classification system used in the public database (available at http://www.hprd.org). The majority of the proteins identified in our research (Tables S1 and S2) were mainly involved in cell growth and/or in the maintenance of dentin cells (25.6%). The next most significant functions were cell communication and signal transduction (15.2%), protein metabolism (14.9%), and metabolism and energy pathways (10.4%). In addition, other significant protein functional groups were related to immune response (9.7%), transport (6.9%), and regulation and nucleic acid metabolism (3.5%). Some proteins were not characterized by the database or had an unknown function (10.4%).

We found that the most significant proportion of human dentin proteins played roles in the growth or the maintenance of dentin cells. This is different from the results of another study, where metabolic enzymes were found to comprise one of the largest groups of human dentin proteins (6). This discrepancy might be explained by the different proteomic approach used in this study, of multiple extraction procedures, and, as a result, better extraction of the proteins from dentinal tubules.

It is obvious that the function of dentin lies not only in providing a support for tooth enamel, the hardest tissue in the human body, but also in playing a role in the human immune-defense system. A significant proportion of the protein in dentin is involved in protein metabolism, which means that the tooth is a fairly active tissue.

This study may also help researchers to compare the proteomes between individual groups of subjects using quantitative proteomics. Comparing the proteomes of a population with various tooth-health characteristics could reveal differences in the presence or abundance of characteristic protein markers.

In conclusion, this study effectively released proteins from the calcified dentin sample as a result of the application of two complementary destruction steps: denaturation by a chaotrophic agent (guanidine) followed by demineralization (with EDTA). This technique, coupled with 2D-GE and nLC-MS/MS, provided the broadest and most comprehensive protein map of human dentin proteins to date. We were able to identify 289 proteins overall, 90 of which had not been previously detected in human dentin. Interestingly, nine 'putative' or 'hypothetical' proteins were identified, mainly classified as immunoglobulins. Those nine proteins were detected, for the first time, as proteins in the human body.

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Conflicts of interest – All authors declare that there are no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of proteins detected by two-dimensional gel electrophoresis from human dentin tissue and identified by mass spectrometry.

Table S2. List of keratins and other possible contaminants detected by two-dimensional gel electrophoresis from human dentin tissue and identified by mass spectrometry.

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