Journal of
PERIODONTAL RESEARCH

J Periodont Res 2011; 46: 111–117

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01320.x

Proteomic identification of proteinase inhibitors in the porcine enamel matrix derivative, EMD®

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Zilm PS, Bartold PM. Proteomic identification of proteinase inhibitors in the porcine enamel matrix derivative, EMD®. J Periodont Res 2011; 46: 111–117. © 2010 John Wiley & Sons A/S

Background and Objective: The porcine enamel matrix derivative, EMD®, which is the active component of Emdogain®, is used widely in periodontics because of its ability to promote the regeneration of soft and hard tissues and to reduce inflammation. Previous studies have used indirect methods to explain its angiogenic and proliferative effects on cells associated with wound healing. In this study we used proteomic techniques to identify proteins in EMD other than amelogenins.

Material and Methods: Proteins in EMD were separated by two-dimensional gel electrophoresis and were identified using mass spectrometry. Proteomic results were validated by western blot analysis of Emdogain.

Results: Fourteen proteins of porcine origin were identified and included the serine and cysteine proteinase inhibitors alpha1-antichymotrypsin and fetuin A, respectively. Alpha1-antichymotrypsin is an acute-phase factor that has been reported to indirectly down-regulate the expression of the gelatinase MMP-9. Fetuin A, a major glycoprotein component of bone and teeth, is a potent inhibitor of ectopic calcification of vascular and soft tissues and has been implicated in both osteogenesis and bone resorption. It also facilitates plasma membrane repair in damaged fibroblasts.

Conclusion: EMD contains a number of high-molecular-weight compounds which include the proteinase inhibitors, fetuin A and alpha1-antichymotrypsin.

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Key words: alpha1-antichymotrypsin; fetuin A; proteomics; enamel matrix derivative®; emdogain®

Accepted for publication July 22, 2010

Emdogain® (Straumann Australia Pty Ltd, Melbourne, Vic., Australia). a product composed of an alginate carrier (propylene glycol alginate) and porcine enamel matrix derivative (EMD®; Straumann Holding AG, Basel, Switzerland), is widely used in the treatment of periodontal damage resulting from periodontitis and has been shown to promote tissue regeneration and to decrease inflammation following periodontal surgery (1–6). Emdogain has been found to contain a number of low-molecular-weight proteins (mainly amelogenins) that are associated with cementogenesis and osteogenesis during tooth development (3,7–9). However, the presence of enamel matrix proteins alone is unlikely to explain the wide diversity of outcomes attributed to EMD treatment (10,11). A number of studies have reported on the biological effects of EMD. These effects include stimulation of proliferation of mesenchymal cells and epithelial cell rests of Malassez, enhanced wound healing, enhanced extracellular matrix synthesis and the regulation of type 1 collagen mRNA, bone sialoprotein, osteocalcin and osteopontin by periodontal cells (6,12–16).

Although many studies have described the regulatory effects of EMD, only a few have attempted to identify the regulatory proteins or growth factors that may be present. Maycock *et al.* (9) used sodium dodecyl sulphate–polyacrylamide gel

electrophoresis (SDS-PAGE), western blotting and zymography to search for previously unidentified components in EMD, and reported the presence of metallo-endoprotease and serine-protease activity. Subsequently, Suzuki *et al.* (5) fractionated EMD gel using size-exclusion chromatography and used a reporter assay to detect the activity of the osteoinductive factors, bone morphogenic protein and transforming growth factor-β, and concluded that they contributed to the induction of biomineralization by EMD. However, in neither study were the proteins or growth factors present positively identified. The secretion of metallo-endoproteases by osteoblasts plays a crucial role in tissue remodelling by degrading the extracellular matrix (2), but conflicting evidence has been reported showing both the stimulation and down-regulation of the potent collagenase, MMP-1, in osteoblasts following exposure to EMD (2,14). However, potential regulators of MMP-1 activity have not been identified.

For a number of years, proteomics, using two-dimensional gel-electrophoresis (2DE), mass spectrometry (MS) and database searches, has proved a powerful methodology with which to determine the composition of complex mixtures of proteins (17,18).

In light of the inconclusive results obtained in determining the composition of EMD, in this study we used a proteomic approach to identify specific components of EMD other than amelogenins.

Material and methods

Chemicals/equipment

For western blotting, Emdogain (Straumann Australia Pty Ltd) was purchased commercially. For SDS-PAGE and 2DE, EMD was supplied by Straumann Holding AG as a lyophilized preparation free of the alginate carrier. DeStreak reagent was purchased from GE Healthcare (Fairfield CT, USA); all other chemicals used in SDS-PAGE and 2DE, solubilization buffer, IPG strips, iso-electric focusing (IEF) equipment, and densitometry and image-analysis software (PDQuest version7.2) used for proteomic analysis, were purchased from BioRad Laboratories (Hercules CA, USA).

Preparation of EMD for proteomic analysis

Lyophilized EMD was suspended directly in a solubilizing solution (30 mg/mL) containing 5 M electrophoresis-grade urea, 2 M thiourea, 40 mM Tris buffer, 2 mM tri-butyl phosphine, 2% CHAPS, 2% SB 3–10 and 0.2% (w/v) ampholytes. Samples were left for 1 h at room temperature and then the solution was aspirated with a fine-gauge needle. Samples were then clarified by centrifugation (20,000 *g*, 60 min, 15°C) to remove nonsoluble material and stored at −80°C.

2DE

Protein quantification was performed using an RC DC protein assay kit (BioRad Laboratories), in accordance with the manufacturer's instructions. IEF was performed on 11-cm precast IPG strips with pH ranges of 3–6, 4–7 and 7–10, using a Protean IEF cell. Briefly, 0.35 mg of protein was cup loaded (anode end) onto an 11-cm IPG strip that had been passively rehydrated for 20 h in 0.2 mL of solubilization buffer containing DeStreak reagent (15 mg/mL) instead of tri-butyl phosphine. IEF was run using a customized method. Briefly, the voltage was gradually increased, using linear ramping, to 4000 V over 8.5 h as follows: 150 V for 1 h, 300 V for 3 h, 600 V for 1.5 h and 1200 V for 1.5 h, followed by rapid ramping to 4000 V over 1.5 h. Focusing occurred at 8000 V/h with a 50 μA per strip current limit and the temperature was maintained at 20°C. After 8000 V/h had been achieved, the voltage was maintained at 500 V until IPG strips were removed and stored at −20°C. The IPG strips were subjected to a two-step equilibration, as described by Gorg *et al.* (19). Polyacrylamide gels (12% T, 3.3% C, 0.1% SDS, 375 mM Tris/HCl, pH 8.8) were cast without a stacking gel in a Protean II XL casting chamber. Separation of proteins in the second dimension was carried out using a Protean II XL gel system. The gels were resolved (30 mA/gel) in a Tris-glycine tank buffer (20) until the dye front reached the bottom of the gel. The gels were stained in a solution containing 0.025% (w/v) Coomassie Blue R-250, 40% (v/v) methanol and 7% (v/v) acetic acid, and destained in a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid. The stained gels were scanned using a GS-800 densitometer operated by the software program PD-Quest (BioRad Laboratories). Separation of EMD by 2DE was performed in duplicate.

One-dimensional electrophoresis (SDS-PAGE)

Lyophilized EMD was dissolved in a solution containing 5% (w/v) SDS, 87% (w/w) glycerol, 0.05% (w/v) Bromophenol blue, Tris–HCl (0.063 M) and 3% (v/v) 2-mercaptoethanol, and the proteins were separated by SDS-PAGE using discontinuous gels (12% T, 3.3% C resolving gel and 6% stacking gel) (20).

Protein identification by MS

Protein 'spots' were excised from each gel and placed into 1.5-mL capped tubes (Eppendorf, Hamburg, Germany). The spots were destained with 50% acetonitrile (ACN) and digested with 100 ng (10 ng/μL in 5 mM ammonium bicarbonate) of modified trypsin (sequencing grade; Promega, Madison, WI, USA) per sample. Tryptic peptides were extracted from the gel pieces with 50% ACN/0.3% formic acid (FA) in water. The volumes of the final samples were reduced from approximately 120 μL to approximately 1 μL by vacuum centrifugation. The peptides were then diluted to approximately 5 μL with FA30 [0.1% FA/ACN (7:3, v/v)].

MALDI-MS and MS/MS

One microliter of each digested protein sample was applied to a 600 μm AnchorChip (Bruker Daltonics GmbH, Bremen Germany), following the method of Zhang *et al.* (21). Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF)

mass spectra were acquired at random locations over the matrix surface spot using a Bruker ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics GmbH) in reflectron mode and controlled by flexControl software (version 3; Bruker Daltonics GmbH). External calibration was performed using peptide standards (Bruker Daltonics GmbH).

Between three and six of the most highly abundant sample ions (no trypsin or keratin) were selected for MALDI-TOF/TOF analysis, which was performed in LIFT mode using the sample spot on the target.

Mass spectra data acquired by MS and MS/MS were analysed using flexAnalysis (version 3; Bruker Daltonics GmbH) and then exported to BioTools (version 3.1; Bruker Daltonics GmbH), and the MS and corresponding MS/MS data were combined and used to interrogate an in-house Mascot database search engine (version 2.2; Matrix Science: http://www.matrixscience.com) using the following parameters.

Taxonomy: all entries.
Database: NCBI nonredundant download date 2008-02-14.
Enzyme: trypsin.
Fixed modifications: carbamidomethyl.
Variable modifications: oxidation.
Mass tolerance: 50 ppm.
MS/MS tolerance: 0.5 Da.

Protein identification was based upon the molecular weight search (MOWSE) and probability scores generated by the software. If the score was above the threshold, the probability that the match was random was $< p = 0.05$. Based on the combined data, the MS/MS of samples that returned a positive 'hit' were submitted independently to Mascot. The predicted molecular weights and isoelectric points of identified proteins were calculated using 'Compute pI/MW' from the Expert Protein Analysis System (http://au.expasy.org/tools/pi_tool.html).

Liquid chromatography-electrospray ionization MS

Samples that did not give sufficient spectra, upon MALDI-MS, for accurate protein identification were further analysed using liquid chromatography–electrospray ionization (μLC-ESI) ion-trap MS. A 2.5-μL volume of the samples containing the protein 'spots' digested with trypsin were diluted with 3% ACN and 0.1% FA in an autosampler vial, and 5 μL of each sample was introduced into an Agilent Protein ID Chip column assembly (a 40-nL trap column with a 0.075×43 mm C-18 analytical column) housed in an Agilent HPLC-Chip Cube Interface connected to an HCT ultra 3D-ion-TRAP mass spectrometer (Bruker Daltonics GmbH). After equilibration of the column with 4% ACN/0.1% FA at a flow rate of 0.5 μL/min, the samples were eluted over 32 min with an ACN gradient of 4–31%.

Ionizable species ($300 < m/z < 1200$) were trapped, and one or two of the most intense ions eluted were fragmented by collision-induced dissociation. Peak detection of MS and MS/MS spectra was performed using Data Analysis (version 3.4; Bruker Daltonics GmbH), the data were imported into Biotools and the MS/MS data were searched as previously described (MALDI-MS and MS/MS), but with an MS mass tolerance and an MS/MS tolerance of 0.3 and 0.4 Da, respectively; peptide charges were 1+, 2+ and 3+, and missed cleavages were set to 1.

Western blotting

Emdogain (30 mg/mL) was purchased commercially (Ref: 075.102, Lot R3389; Straumann Australia Pty Ltd) and 0.25 mL was diluted into 10 mL of phosphate-buffered saline (0.1 M, pH 6.5). A 1-mL aliquot was removed and added to an equal volume of 2× concentrated SDS-PAGE buffer (20) to give approximately 0.38 mg/mL of protein.

Approximately 4 μg of protein was separated on a 12% T 3.3% C SDS-polyacrylamide gel (20). The proteins were then electrotransferred to a Hybond™-P poly(vinylidene difluoride) transfer membrane (GE Healthcare). Transfer membranes were blocked in 1% (w/v) Tris-buffered saline (TBS) containing 25 μg/mL of membrane-blocking agent (RPN2125V; GE Healthcare), 1% (w/v) bovine serum albumin (Sigma-Aldrich St. Louis, MO. USA) and 0.1% (v/v) Tween 20. The membranes were then probed overnight, at 4°C, with 1 μg/mL of polyclonal goat anti-(human fetuin) (Rockland Inc., Gilbertsville, PA, USA), and 1 μg/mL of monoclonal mouse anti-(human alpha1 antichymotrypsin) (Abcam, Sapphire Bioscience Pty. Ltd, NSW, Australia) primary antibody in TBS containing 1% bovine serum albumin and 0.1% Tween 20. After washing in TBS containing 0.1% Tween 20, the membranes were incubated with the appropriate secondary antibody, either anti-(mouse IgG) alkaline phosphatase (ALP)-conjugated (Chemicon Australia Pty. Ltd., Melbourne, Australia), or anti-(goat IgG) ALP-conjugated (Chemicon International, Temecula, CA, USA) at 1 μg/mL. Proteins were detected using 1 mL of ECF substrate (RPN5785; GE Healthcare) and scanned using a Typhoon 9410 variable-mode imager (Molecular Dynamics Inc., Sunnyvale, CA, USA). Western blotting of Emdogain was performed in triplicate.

Results

Figure 1B shows the 2DE and SDS-PAGE separation of proteins extracted from the lyophilized EMD. SDS-PAGE gels resolved many proteins, the most abundant of which had a molecular mass of < 25 kDa (Fig. 1). The separation of complex mixtures of proteins, using one-dimensional electrophoresis Fig. 1A resolved many proteins with relatively poor resolution.

Using the 2DE protocol, proteins were resolved in the first dimension, over a pI range of 3–10, by using 11-cm IPG strips with pH ranges of 3–6, 4–7 and 7–10. As there was some overlap between the pI ranges of the individual 2DE gels, some protein spots were represented in more than one gel. Fourteen of the most abundant (based on the intensity of staining) and well-resolved spots were excised from the gels and, following in-gel digestion, were initially identified by MALDI-TOF/TOF spectrometry, (Table 1). If MOWSE scores were below or close to

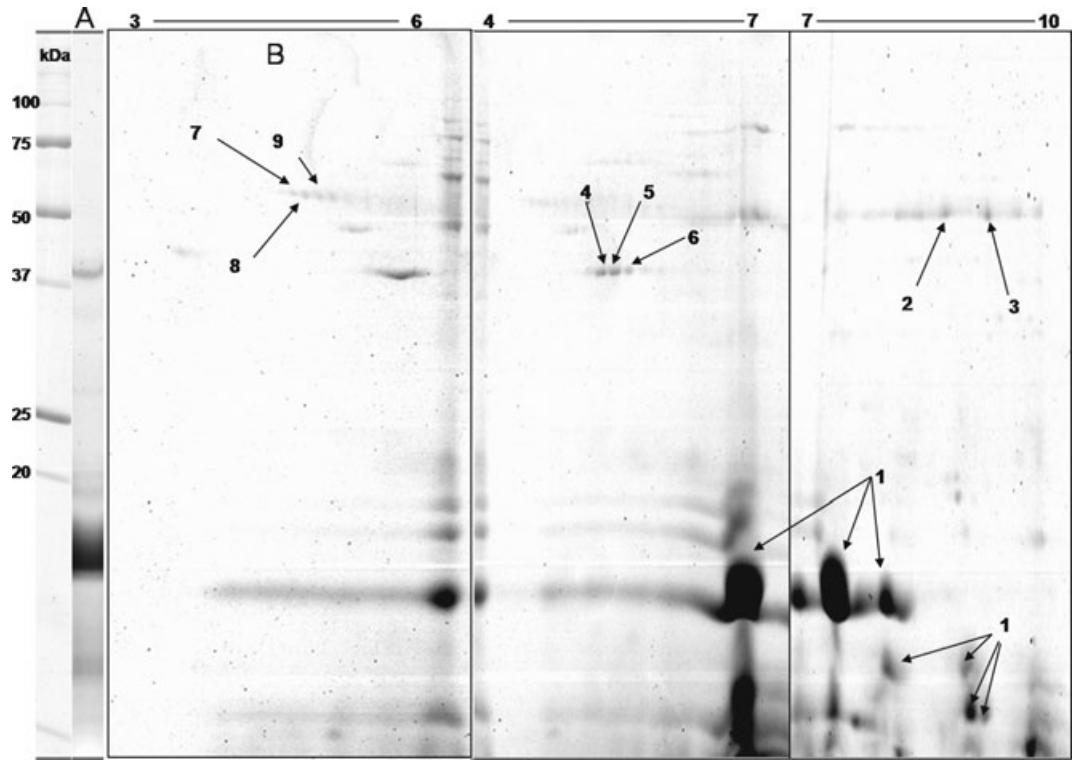

Fig. 1. Separation of proteins in lyophilized EMD and visualization of protein bands by staining with Coomassie Blue R250. (A) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (B) Two-dimensional gel-electrophoresis (2DE). The horizontal axes represent the isoelectric focusing gradient of each gel and the vertical axes represent molecular mass (kDa). Molecular mass standards for 2DE and SDS-PAGE gels are shown on the pI 3–6 and 4–7 gels, and on SDS-PAGE gels, respectively.

the cut-off threshold, individual peptide ions were analysed using ESI ion-trap MS. Protein identifications were also supported by comparing observed and predicted pI and molecular mass values. Most proteins showed relatively small variations in molecular mass and pI values (Table 1), the exception being the glycoproteins alpha1-antichymotrypsin and fetuin A. All proteins were identified as porcine and their identities are summarized in Table 1. Five of the samples produced sufficient spectra using MALDI-MS or MS/MS, whereas the remaining were identified using ESI ion-trap MS or MS/MS.

2DE was used to increase protein resolution and to reduce the chances of obtaining overlapping spots (22). Of the fourteen proteins identified, only two (spots 3 and 9, Table 1) were found to contain two proteins. The relative abundance of each protein was quantified by calculating the exponentially modified protein abundance index (emPAI) score from the ESI-MS/MS data (23). Spot 3 contained immunoglobulin components, with the heavy chain being predominant. Spot 9 contained alpha1-antichymotrypsin 3 and fetuin A, with the former having an emPAI score approximately twice as high as that of the latter (Table 1). The relative proximity of spots 8 and 9 (both identified as containing fetuin A) suggests that horizontal streaking during IEF may have contributed to the incomplete separation of these proteins.

The relatively low molecular mass of amelogenins, and their anomalous migration under electrophoretic conditions provides an explanation for their absence in any of the spots excised from the gels (24).

In separate experiments aimed at validating the proteomic results, the presence of fetuin A and alpha1-antichymotrypsin in commercially available Emdogain was demonstrated by western blot analysis. Antibodies raised against human alpha2-HS-glycoprotein (fetuin) and alpha1-antichymotrypsin reacted with the Emdogain preparation, giving bands that corresponded to the expected molecular mass range of the native proteins (Fig. 2).

Discussion

As stated earlier, a large number of studies have reported the effects of EMD on healing and regeneration of periodontal ligament and alveolar bone following surgery (6,14–16,25). In attempts to fully understand the role of EMD, many studies have examined the effects of EMD on regenerative cells, such as osteoblasts (12), while others have addressed the composition of EMD (5,9).

In the present study, we identified a number of nonamelogenin proteins, including the serine protease inhibitor, alpha1-antichymotrypsin in EMD. Alpha1-antichymotrypsin is an acute-phase protein produced by the liver in response to trauma and belongs to the serine proteinase inhibitors (serpins) superfamily (26). Serpins are associ-

Table 1. Proteins identified in lyophilized porcine enamel matrix derivative, EMD

Spot #[a]	Protein identification[b]	Organism[c]	NCBI accession number[d]	Mascot score/ cut-off[e]	% seq. MS[f]	% seq. MS/MS[f]	No. of unique peptides[g]	emPAI[h]	Pred. MW[i]	Pred. pI[j]	Obs. MW[i]	Obs. pI[j]
1*	Ameloblastin precursor	*Sus scrofa*	gi47522894	49/41 114/41 103/41 63/41 61/41 81/41		3 7 7 3 3 3	1 2 2 1 1 1		45.0	5.5	12–20	7–8.5
2	Immunoglobulin gamma chain	*Sus scrofa*	gi164503	213/70	23.2	5.1		–	52.2	7.7	52	8.5
3*	Immunoglobulin heavy chain variable region Immunoglobulin gamma chain	*Sus scrofa*	gi54888402 gi45269029	217/55 188/55		36 13	5 6	0.58 0.13	45.1	5.6	48	5.4
4	Cytoskeletal beta actin	*Sus scrofa*	gi45269029	226/71	45	12.5		–	45.1	5.6	48	5.5
5	Cytoskeletal beta actin	*Sus scrofa*	gi45269029	386/71	34	10		–	45.1	5.6	48	5.5
6	Cytoskeletal beta actin	*Sus scrofa*	gi45269029	184/70	38	12.5		–	45.1	5.6	48	5.5
7	Mitochondrial ATP synthase H+ transporting F1 complex beta subunit	*Sus scrofa*	gi89574051	271/70	47.6	7.6		–	47.1	4.9	50	5
8*	Fetuin A	*Sus scrofa*	gi231467	164/55		14	3	–	39.2	5.5	63	5.1
9*	Alpha1-antichymotrypsin 3 Fetuin A	*Sus scrofa*	gi9968807 gi231467	221/55 141/55		30 14	6 3	0.51 0.28	22.9 39.2	5.8 5.5	63 63	5.3 5.1

[a]Spot numbers shown in Fig. 1.
[b]Protein names were obtained from annotations in the NCBI database.
[c]The species from which the protein was identified.
[d]National Centre for Biotechnology Information.
[e]For electrospray ionization-ion trap (ESI-IT) matching, the protein (combined ion) score with cut-off score for individual ions was used to indicate identity or extensive homology ($p < 0.05$). For matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF)/TOF matching, the protein score with cut-off score was used to indicate a positive protein identification ($p < 0.05$).
[f]Sequence coverage identified from mass spectrometry (MS)/MS data or MS data, expressed as the number of amino acids spanned by the assigned peptides divided by the sequence length.
[g]The number of unique peptides found to match the identified protein, not including different charge states or modification states of the same peptide.
[h]The exponentially modified protein abundance index (emPAI value) as a measure of relative quantification; scores were only calculated when more than one protein was identified in the same spot. emPAI scores were not calculated for MALDI data (as indicated by a dash).
[i]Predicted molecular weight (MW) determination derived from the (unmodified) amino acid sequence (http://au.expasy.org/tools/protparam.html) and observed MW estimated from two-dimensional gel-electrophoresis (2DE) using MW markers as a reference.
[j]Predicted isoelectric point determination derived from the (unmodified) amino acid sequence (http://au.expasy.org/tools/protparam.html) and observed pI estimated from 2DE gels.
*Proteins identified using ESI-IT MS. Other proteins were identified using MALDI-MS.

ated with the control of many physiological functions, including blood coagulation and wound healing (26). Alpha1-antichymotrypsin has been shown to potently inhibit the zymogen activation of pro-MMP-9 in human and rodent skin (26). MMP-9 (gelatinase B) is commonly found at sites of chronic wounds and is commonly associated with inflammatory diseases, such as periodontitis (27).

During synthesis, alpha1-antichymotrypsin undergoes post-translational glycosylation at specific asparagine residues. The observed differences in predicted molecular mass and pI values, seen in Table 1, are a result of the incomplete protein sequence deposited in the NCBI database. We were therefore unable to deduce the number of glycosylation sites or the correct molecular mass or pI. Intact human alpha1-antichymotrypsin has been shown to have mobility (approximately 63 kDa) similar to that shown in Fig. 1 (28).

The presence of alpha1-antichymotrypsin in EMD may potentially go some way towards explaining its wound-healing properties; however, it should be emphasized that in light of its medical use, and the large number of studies reporting the healing effects

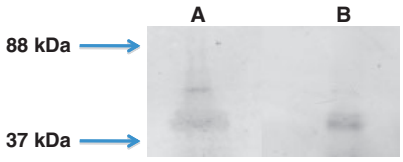

Fig. 2. Western blot of Emdogain. Western blots were probed with anti-human alpha1-antichymotrypsin (A) or fetuin (B) primary antibodies. Molecular mass markers are indicated on the left. The results are representative of duplicate experiments.

of alpha1-antichymotrypsin, the hypothesis that alpha1-antichymotrypsin contributes to the effectiveness of Emdogain remains speculative.

This study showed that the cysteine proteinase inhibitor, fetuin A, is also present in EMD. Fetuin A is a globular protein that constitutes 45% of the protein in fetal calf serum and is the major noncollagenous protein found in bone, teeth and other ectopic calcified tissues (29). Its appearance in EMD may not be surprising as it has homologues in sheep, pig, goat, rats and humans, and is expressed during embryogenesis in multiple tissues and is produced by the tongue and placenta (30). It is also an acute-phase protein that is released by the liver and has been shown to be up- and down-regulated in certain species (29). Alpha2-HS-glycoprotein is the human homologue of fetuin A and has been shown by SDS-PAGE to have a molecular mass close to 63 kDa. It has multiple post-translational sites that are glycosylated and phosphorylated, and this explains the divergence between the theoretical and observed molecular mass values shown in Table 1 (29). It appears to be important in a number of biological functions, including osteogenesis and bone resorption (31), regulation of insulin activity (32), regulation of cytokine expression (33), repair of cell membranes (34) and the decalcification of soft tissues (29).

The presence of the alginate carrier in Emdogain interfered with the separation of the protein by 2DE; however, to determine whether the proteomic results could be independently validated, western blots of Emdogain were probed with anti-(human fetuin A) and anti-(human alpha1-antichymotrypsin) primary antibodies. The results shown in Fig. 2 indicate that both were present in commercially available Emdogain preparations. It was noted that the observed molecular mass values for both alpha1-antichymotrypsin and fetuin A were lower than those seen on the 2DE separation of EMD. As stated previously, the incomplete protein sequence of alpha1-antichymotrypsin deposited in the NCBI database made it impossible to determine its theoretical molecular mass. However as fetuin A also showed a reduced molecular mass, it is likely that the different denaturation protocols of 2DE and SDS-PAGE may have highlighted differences in the levels of denaturation and SDS binding.

We also identified a number of immunoglobulin, cytoskeletal and mitochondrial components (Table 1) in EMD. The contributing role, if any, of these proteins to the biological effects of EMD are speculative and they may simply represent proteins that are present in a relatively unpurified fraction of enamel matrix. The similarities in observed and predicted molecular mass and pI, and their porcine origin, serve to illustrate the reliability of the data.

Of all the small-molecular-mass enamel matrix proteins believed to be present in EMD, only ameloblastin was identified in a number of spots with a low molecular mass (Table 1). This contrasts with other studies which suggest that EMD contains the glycoprotein, amelogenin, and nonamelogenin proteins (e.g. ameloblastin, enamelin) at a ratio of approximately 90:10 (35,36). In a proteomic study comparing the protein composition of porcine soft- and hard-enamel matrices, Yamakoshi *et al.* (37) reported that, in hard enamel, the acidic glycoproteins enamelin and the 29-, 27-, 15-, 13-, 8- and 6-kDa C-terminal fragments of ameloblastin were absent. The gradual loss of these glycoproteins, caused by the presence of serine proteases, during enamel development is naturally associated with the increase in enamel mineralization. The presence of C-terminal fragments of ameloblastin may be responsible for the number of spots within this molecular mass range that were identified as ameloblastin (Fig. 1).

Determination of the amino sequence of porcine amelogenins has revealed a high degree of homology with amelogenins from other species. The porcine amelogenin polypeptides leucine- and tyrosine-rich amelogenin protein (LRAP and TRAP) are reported to have a molecular mass of 5–7 kDa and are derived from a precursor protein with a molecular mass of 18–25 kDa (38). The molecular mass of bovine amelogenins has also been shown to be heterogeneous, and their separation by SDS-PAGE has been described as anomalous (24). Considering that the proteomic techniques cannot be guaranteed to separate all proteins, the conditions used may not have resolved amelogenins as they would have migrated off the gel during SDS-PAGE.

To our knowledge this is the first study to positively identify the presence of serine and proteinase inhibitors in EMD; the diverse regulatory functions of these proteins and their role in wound healing, although speculative, may help to explain the clinical effects of EMD.

Acknowledgements

We acknowledge the contribution of staff at the Adelaide Proteomics Centre, The University of Adelaide. This study was supported by funding from the National Health and Medical Research Council of Australia, the Australian Dental Research Foundation and Straumann Holding AG (Basel, Switzerland). Lyophilized EMD® was supplied by Straumann Holding AG (Basel, Switzerland). Emdogain® was purchased from Straumann Australia Pty Ltd. Both authors are inventors for a patent application (Purified EMD Protein composition) filed with the Swedish patent and registration office.

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