RESEARCH REPORTS
Biological

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J Dent Res 89(10):1160-1065, 2010

Surface Integrity Governs the Proteome of Hypomineralized Enamel

ABSTRACT

Growing interest in the treatment and prevention of Molar/Incisor Hypomineralization (MIH) warrants investigation into the protein composition of hypomineralized enamel. Hypothesizing abnormality akin to amelogenesis imperfecta, we profiled proteins in hypomineralized enamel from human permanent first molars using a biochemical approach. Hypomineralized enamel was found to have from 3- to 15-fold higher protein content than normal, but a near-normal level of residual amelogenins. This distinguished MIH from hypomaturation defects with high residual amelogenins (amelogenesis imperfecta, fluorosis) and so typified it as a hypocalcification defect. Second, hypomineralized enamel was found to have accumulated various proteins from oral fluid and blood, with differential incorporation depending on integrity of the enamel surface. Pathogenically, these results point to a pre-eruptive disturbance of mineralization involving albumin and, in cases with post-eruptive breakdown, subsequent protein adsorption on the exposed hydroxyapatite matrix. These insights into the pathogenesis and properties of hypomineralized enamel hold significance for prevention and treatment of MIH.

KEY WORDS: Molar/Incisor Hypomineralization (MIH), protein profiling, clinical proteomics, mechanisms of pathogenesis.

DOI: 10.1177/0022034510375824

Received October 1, 2009; Last revision April 7, 2010; Accepted April 9, 2010

While this article was in press, others reported finding serum proteins in MIH and normal enamel but their origins (i.e., pre-eruptive vs. post-eruptive) were not investigated (Farah RA, Monk BC, Swain MV, Drummond BK, Protein Content of Molar-Incisor Hypomineralisation Enamel, *Journal of Dentistry* (2010), doi:10.1016/j.jdent.2010.04.012).

A supplemental appendix to this article is published electronically only at http://jdr.sagepub.com/supplemental.

INTRODUCTION

Developmental defects of enamel are disturbingly prevalent and costly, potentially afflicting over 10% of the population with multiple burdens including dental pain, disfigurement, and increased caries risk (Hall, 1994; Arrow, 2008). Classically, attention has centered on the rare genetic disorders termed 'amelogenesis imperfecta' (AI) and on dental fluorosis, a widespread defect acquired from excessive fluoride intake. Over the past decade, however, another acquired defect has been causing increasing concern to clinicians worldwide. Primarily disrupting mineralization of permanent first molars and incisors, this condition is commonly termed Molar/Incisor Hypomineralization (MIH) (Weerheijm, 2004).

MIH typically affects 10-20% of children and brings with it substantial treatment needs, often extending to extraction and orthodontics. Restorative treatment is frequently compromised, because MIH enamel is soft, porous, and poorly delineated from normal tooth tissue (Jalevik and Klingberg, 2002; Mejàre *et al*., 2005; William *et al*., 2006; Arrow, 2008). MIH is thought to be acquired *via* a multifactorial, systemic disturbance of the enamel-forming cells. However, other than being dissociated from fluoride and linked to illness during infancy, the cause of MIH remains a mystery (Whatling and Fearne, 2008; Arrow, 2009; Crombie *et al*., 2009; Laisi *et al*., 2009). It is reasonable to anticipate that MIH will become preventable once its causes are unraveled, given past successes with tetracycline- and fluoride-induced dental defects (Hall, 1994; Tredwin *et al*., 2005).

Little is currently known about the protein composition of MIH enamel, which is troubling from etiological and clinical viewpoints. Addressing clinically relevant properties of MIH enamel, biophysical studies revealed unusual characteristics that were unexplainable by mineral behavior alone (Mahoney *et al*., 2004; Xie *et al*., 2009). Pathogenically, it is unknown whether the protein-rich enamel matrix is secreted and matured normally in MIH, thus hindering enquiry into the role of cellular injury. In this study, we sought to characterize the protein composition of MIH enamel, hypothesizing that it is abnormal akin to AI and fluorotic enamel (Wright *et al*., 1996, 1997; Takagi *et al*., 1998). We addressed known 'players' (*i.e*., amelogenins, which comprise > 90% of protein in developing enamel) as well as unknowns *via* proteomics. Our focus was on first permanent molars, which are of greater dental concern than incisors (*cf.* prevalence, caries risk, treatment challenges). Hereafter we refer to such defects as idiopathic Molar Hypomineralization (iMH).

MATERIALS & METHODS

Specimens

Tissue specimens were obtained with appropriate ethical approvals and stored at -80°C. MIH/iMH was diagnosed in 7- to 10-year-old children according to standard criteria (Weerheijm, 2003). After extraction, iMH teeth (n = 5, containing 22 lesions) were water-rinsed to remove visible blood, then blotted dry and stored frozen immediately. Whole saliva, stimulated by the donor chewing on wax, was clarified by centrifugation (20,000 *g,* 5 min) before storage. Serum and erythrocytes were prepared conventionally from blood of 6-day-old rats (Sprague-Dawley). Secretory enamel matrix was isolated from developing rat teeth as before (Hubbard, 1996) except using 5-day-old first molars.

Profiling of Enamel Proteins

We collected overt iMH lesions from freshly thawed specimens by scraping with a scalpel, taking care to avoid carious enamel and dentin. Visibly normal enamel was sampled from the same teeth with a slowly rotating dental bur (No. 6). Immediately afterward, enamel samples (2-5 µL packed vol) were suspended in 10% trifluoroacetic acid (10 vol, 10 min at room temperature with vortexing and bath sonication), then centrifuged (20,000 *g,* 4°C, 5 min) to sediment acid-insoluble protein. Pellets were solubilized in gel-loading buffer containing 2% SDS and 100 mmol/L dithiothreitol (Hubbard, 1996), with additional protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L benzamidine, 5 µg/mL pepstatin, 5 µg/mL leupeptin) where indicated. SDS extracts were quantified by dot blotting with Amido Black and subjected to mini-SDS-PAGE with Coomassie Blue staining or immunoblotting (Hubbard, 1995). Amelogenin antiserum was raised conventionally in rabbits, with recombinant mouse amelogenin (a gift from Alan Fincham) as immunogen.

Proteomic Analysis

Gel bands were subjected to trypsinolysis and tandem mass spectrometry as before (Mangum *et al*., 2006), except with an ion-trap instrument incorporating chip-based nanospray (Chip-LC/MSD XCT, from Agilent Technologies, Santa Clara, CA, USA). Proteins were identified by means of the MASCOT search engine and SwissProt human database, with strict acceptance criteria [minimally, two sequence tags (Mangum *et al*., 2006)].

Mineral-binding Assays

We prepared mock oral fluid by empirically spiking saliva with serum and erythrocyte lysate so that major proteins from all 3 components were similarly abundant (Fig. 4B). To assay protein binding, we incubated oral fluid with 0.1 vol hydroxyapatite (from Sigma, St. Louis, MO, USA) or iMH enamel for 60 min at 20°C, then centrifuged it (2000 *g,* 2 min). After being washed in 3 vol 20 mM Tris-HCl (pH 8.0), the pellet was extracted with trifluoroacetic acid and SDS as described above for enamel.

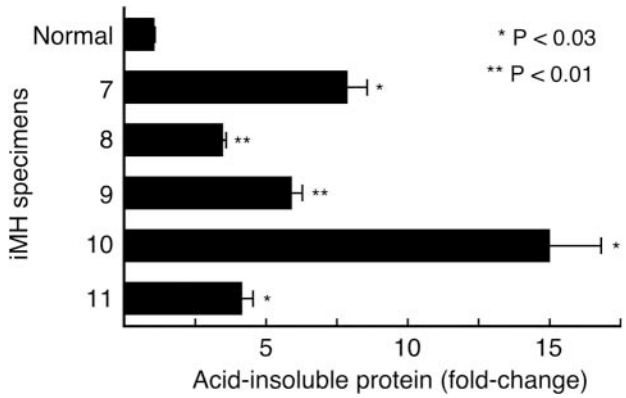

Figure 1. The protein content of iMH enamel is abnormally high. Acid-insoluble proteins were extracted from normal enamel (normal) and a group of severe lesions exhibiting post-eruptive breakdown (specimens 7–11), then quantified by densitometric dot-blot analysis. Mean values (± SD) are shown for duplicate assays, each done at varied loads to ensure quantitative linearity ($r^2 > 0.95$). As indicated, all iMH specimens differed significantly from normal when compared pairwise by Student's *t* test (homoscedastic, two-tailed). An albumin standard was used to derive absolute protein levels from these data (see the text).

RESULTS

iMH Enamel is Enriched with Non-amelogenin Proteins

Profiling of enamel proteins has provided useful insights into the pathogenesis of fluorosis and AI. Moreover, the classification of such hypomineralized lesions into hypomaturation and hypocalcified subtypes (Witkop, 1988) was given a molecular foundation by linking amelogenin levels with clinical properties (Wright *et al*., 1996, 1997; Takagi *et al*., 1998). Accordingly, we investigated unfixed iMH enamel specimens using an SDS-PAGE approach. Unlike normal enamel, iMH enamel gave visible precipitates when dissolved in acid, suggesting a relatively high protein content. Quantification of acid-insoluble protein from 5 severe lesions (Fig. 1) yielded values 3- to 15-fold higher than normal (0.3-1.5% protein w/w). Similarly, SDS-PAGE with Coomassie staining revealed numerous protein bands in iMH enamel, in contrast to barely detectable banding in normal enamel (Fig. 2A). Since amelogenins were undetected (Fig. 2A, 20- to 25-kDa region), immunoblotting was used for higher sensitivity. Anti-amelogenin also failed to detect intact amelogenins in iMH enamel, but degradative fragments were observed in some specimens (Fig. 2B, specimen 11). Amelogenins were undetectable in normal enamel under these conditions (not shown). Quantitative comparison with secretion-phase enamel matrix showed that iMH enamel contained only 0.12% ± 0.06% (± SE, n = 6) the amount of total detectable amelogenins (Fig. 2B, 8- to 25-kDa region). We concluded that iMH enamel is enriched with proteins other than amelogenins.

Body Fluid Proteins Predominate in iMH Enamel

To identify the major protein constituents of iMH enamel, we subjected SDS-PAGE bands to proteomic analysis. A variety of

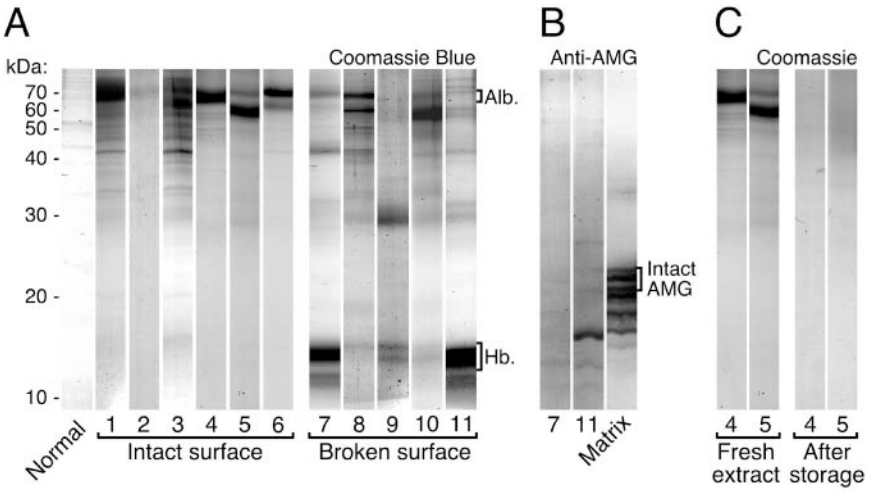

Figure 2. Intact and broken iMH lesions have distinct protein profiles. Acid-insoluble proteins from iMH lesions and normal enamel (normal) were subjected to SDS-PAGE and stained with Coomassie Blue or immunoblotted with amelogenin antibodies (anti-AMG) as indicated. **(A)** Comparison of intact-surface and broken-surface lesions (specimens 1–6 and 7–11, respectively, with loadings equated by wet weight of enamel), showing distinct patterns for the major protein bands. The positions of albumin and hemoglobin are indicated (Alb, Hb). Illustrated sample lanes were composited from 3 first-run gels (*i.e.*, sampled from fresh extracts) de-stained to equivalent levels. Note that the observed variations in "lane color" reflect different levels of unbanded proteins, not differences in staining background. **(B)** Comparison of iMH specimens with secretion-phase enamel matrix from rat, which served as a control for predominantly intact amelogenins (AMG). Specimens 7 and 11 (blotted from the same gels as in A) are representative of lesions with low or appreciable amounts of amelogenin fragments, respectively, and are up-loaded 100-fold relative to the matrix sample (based on wet weight). For quantification reported in the text, cross-immunoreactivity between rat and human amelogenins was normalized with a human amelogenin standard (from Abnova, Taipei City, Taiwan). **(C)** Profiles for two intact lesions, comparing the first gel run using fresh extracts (reproduced from A) with a second run after storage of the same SDS-extracts for 16 wks at -20°C. Note the disappearance of the major bands at 66 kDa (albumin).

proteins was identified (16 distinct gene products), 13 of which are found in saliva and crevicular fluid (Kojima *et al*., 2000; Denny *et al*., 2008). The three others (hemoglobin, albumin, complement C3) are major components of blood (Fig. 3, Appendix Table). Consequently, all major proteins identified in iMH enamel are normally associated with body fluids found intra-orally.

Intact and Broken iMH Lesions Have Distinct Protein Profiles

Given the clinical diversity of iMH lesions (color, consistency, size, surface integrity), we asked whether the different presentations have distinct protein compositions. Appraisal of the protein profiles (Fig. 2A) led us to hypothesize that integrity of the enamel surface had a major influence. Notably, when lesions were grouped as "intact" and "broken", the protein-banding patterns appeared qualitatively similar within each group, but two striking differences were apparent between the groups (Fig. 2A, 12-kDa and 66-kDa regions). The 12-kDa band, which was obvious in broken but not intact lesions, routinely contained hemoglobin as a major component (Fig. 3). Conversely, in intact lesions, the 66-kDa band routinely contained albumin only, unlike broken lesions, where albumin was found infrequently at lower levels.

We also queried stability of the protein profiles, noting evidence of protein degradation (Fig. 3: albumin, complement C3) and the key role of proteolysis in enamel maturation. Indeed, when SDS-solubilized samples from Fig. 2A were re-analyzed after frozen storage, the albumin bands had completely disappeared from intact specimens (Fig. 2C). Broken specimens were largely unaffected, however (not shown). Protease inhibitors had little effect on the profiles of fresh iMH samples when added during the initial SDS-solubilization step (not shown), thus rendering these results consistent with SDS-activated proteolysis (Berger *et al*., 1983). These findings highlighted the risk of artifactual proteolysis, and hence only first-run samples are reported (Figs. 1-3). We concluded that intact and broken lesions consistently have distinct protein profiles, supporting our hypothesis that surface integrity influences the protein composition of iMH enamel.

Protein Composition of iMH Enamel Varies with Surface Integrity

It is known that iMH lesions exhibit subsurface porosity (Jalevik and Noren, 2000), and that albumin and hemoglobin bind avidly to hydroxyapatite (Fargues *et al*., 1998). Accordingly, we posited that oral-fluid proteins permeate iMH enamel and selectively bind to hydroxyapatite crystals, subject to the absence of an intact surface layer. When broken lesions were compared with saliva, serum, and erythrocytes, collective similarities in the protein-banding patterns were found (Fig. 4A). In contrast, intact lesions bore an intriguing resemblance to serum alone. These results accorded with oral-fluid proteins being excluded from intact but not broken lesions. Next, we modeled a broken lesion by exposing hydroxyapatite powder to mock oral fluid (combination of saliva, serum, and erythrocyte extract). Profiling of the hydroxyapatite-bound fraction (Fig. 4B) revealed remarkable similarity to broken lesions (Figs. 2A, 4A). When hydroxyapatite was substituted with powdered enamel made from an intact lesion (*i.e.*, to model breakage of the surface layer), the profile was again similar to that of broken lesions (Fig. 4C). These results indicated that the protein composition of iMH enamel is strongly influenced by integrity of the enamel surface.

DISCUSSION

Given growing concerns about MIH worldwide, a pressing need exists to elucidate the protein composition of hypomineralized

enamel. We have found that iMH enamel has a substantially higher protein content than normal, but a near-normal level of residual amelogenins. This characteristic distinguishes iMH from hypomaturation defects that contain high residual amelogenins [AI, fluorosis (Wright *et al*., 1996, 1997; Takagi *et al*., 1998)] and in turn typifies MIH as a hypocalcification defect. Second, iMH enamel was found to have accumulated various proteins from oral fluid and blood, with differential incorporation depending on integrity of the enamel surface. Pathogenically, these results point to a pre-eruptive disturbance of mineralization involving albumin and, in cases with post-eruptive breakdown, subsequent protein adsorption on the exposed hydroxyapatite matrix. These insights into the pathogenesis and properties of iMH enamel hold significance for the prevention and treatment of MIH.

Our results help to explain the clinical and biophysical properties of iMH enamel. The observed 3- to 15-fold elevation in protein content is similar to reports for AI and fluorosis (2.5- to 30-fold; Wright *et al*., 1996, 1997) and appears sufficient to account for the characteristic mechanical weakness of MIH enamel (Mahoney *et al*., 2004; Xie *et al*., 2009). The low residual content of amelogenins likens iMH enamel to hypocalcified subtypes of AI (Wright *et al*., 1997; Takagi *et al*., 1998). Enamel from the latter disorders is described clinically as markedly softer than normal and friable or cheesy (Witkop, 1988; Takagi *et al*., 1998), which coincides with descriptions of MIH enamel (van Amerongen and Kreulen, 1995; Weerheijm, 2004). At the protein level, iMH enamel appears distinguishable from hypocalcified subtypes of AI and fluorosis, particularly based on its uniquely high content of albumin (Wright *et al*., 1996, 1997; Takagi *et al*., 1998). However, given our findings about proteolytic artefact and surface integrity, such detailed comparisons should await reappraisal of previous conclusions.

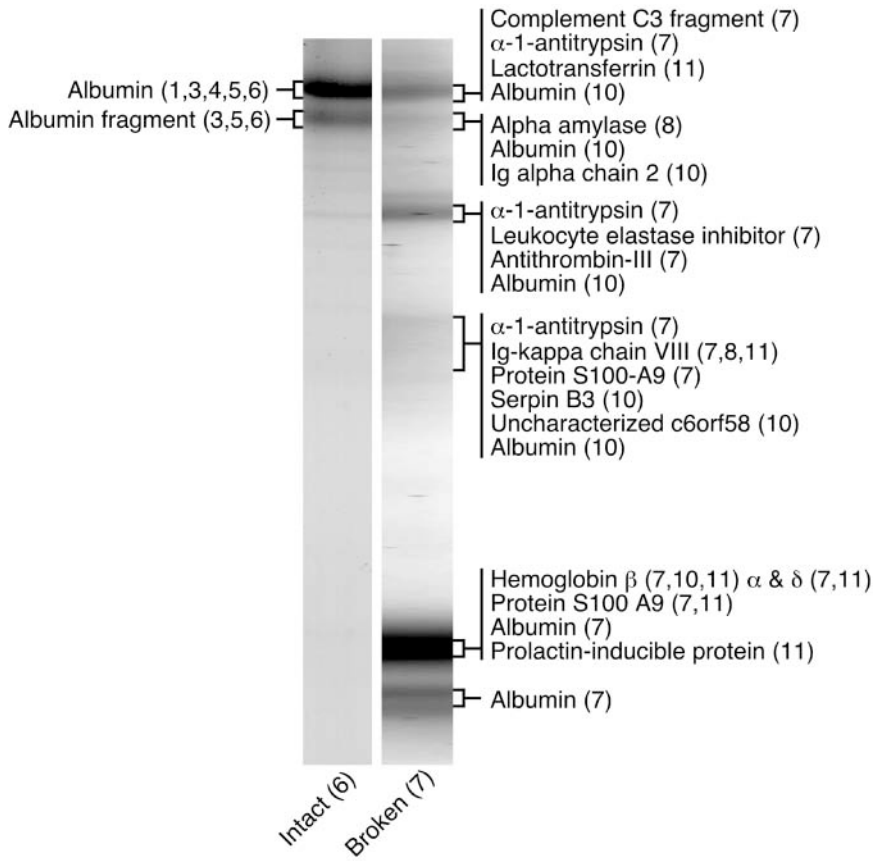

Figure 3. Proteomic analysis reveals numerous body-fluid proteins in iMH enamel. The indicated major gel bands from intact and broken lesions (Fig. 2A, specimens 1–11) were subjected to proteomic identification, as documented more fully in the Appendix Table. The Fig. depicts the proteins identified in each band, and the specimens in which these identifications were made (specimen numbers in parentheses). Gel lanes for specimens 6 and 7 are reproduced from Fig. 2A to illustrate intact and broken lesions, respectively.

Our results also elucidate the pathogenesis of iMH, pointing to pre- and post-eruptive steps that are mechanistically distinct. Pre-eruptively, the normal thickness and low amelogenin content of iMH enamel (< 0.2% of secretion-phase level) indicates that amelogenins are secreted and then removed effectively (albeit incompletely in some cases). It follows that iMH is not a hypomaturation defect primarily related to amelogenin retention. By analogy to hypocalcified AI (Witkop, 1988; Wright *et al*., 1997), attention therefore turns to defective initiation of mineralization. Our profiling indicated that albumin accumulates in iMH enamel despite near-complete removal of amelogenins. Interestingly, it was predicted from animal studies that albumin might inhibit mineralization in enamel defects (Robinson *et al*., 1992, 1994). Widespread suspicions about experimental artefact and conflicting reports have precluded this idea being extended to humans, however (Chen *et al*., 1995; Wright *et al*., 1997; Takagi *et al*., 1998). For the first time, our results substantiate extravasated albumin being accumulated in malforming human enamel. Notably, intact lesions were consistently found to contain albumin, but not numerous oral-fluid proteins with demonstrated hydroxyapatite-binding potential. The observation that albumin but not hemoglobin was prominent may be attributed either to a minor vascular leak of serum rather than whole blood, or to high proteolytic stability of albumin relative to hemoglobin and other blood proteins during enamel maturation. Indeed, albumin is resistant to kallikrein-related peptidase 4 (Takayama *et al*., 2001), a predominant protease involved in amelogenin removal. Further work is required to correlate such albumin leaks to MIH, either causatively or associatively. Our results also imply that another pathogenic step follows post-eruptive (or eruptive) breakdown of the enamel surface, which happens commonly in severe lesions exposed to mastication. This second step involves relatively promiscuous binding of oral-fluid proteins to the exposed hydroxyapatite matrix. It seems plausible that allied variables (*e.g.*, saliva

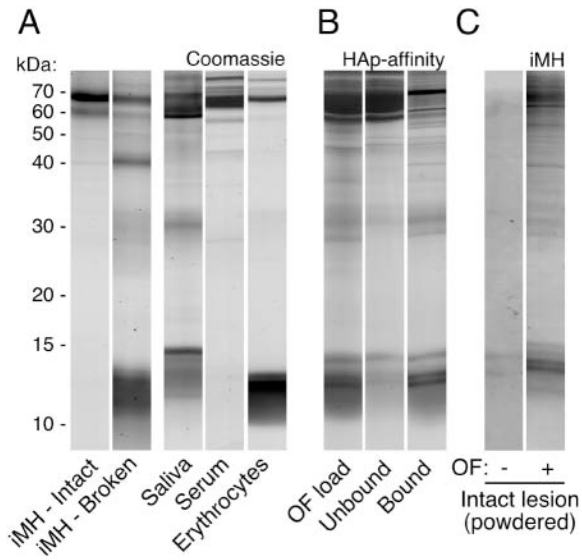

Figure 4. Surface integrity regulates the protein composition of iMH enamel. **(A)** Comparative profiling of iMH enamel and body fluids, showing similarities for intact iMH lesions *vs.* serum and for broken iMH lesions *vs.* saliva and erythrocytes. **(B)** Hydroxyapatite-binding (HAp-affinity) assay, showing that a subset of proteins from mock oral fluid (OF) was preferentially retained (*cf.* differences among the Load, Unbound, and Bound fractions). Note a strong resemblance between the Bound profile and the broken iMH lesion in panel A (specimen 7). **(C)** An equivalent mineral-binding assay to B, but with powdered iMH enamel in place of hydroxyapatite. The profiles show enamel from an intact lesion, before and after exposure to mock oral fluid (+/- OF). Note a resemblance of the protein-bound profile (+) to those of broken lesions and hydroxyapatite in panels A and B. This result indicates that loss of gross structure (including intact surface) leads to a marked change in the protein-binding capability of intact lesions. To legitimize these comparisons, both affinity matrices (particulate hydroxyapatite, iMH enamel) were mortar-ground to equal consistencies (coarse powder) before assay.

composition, dietary protein, enamel porosity) could contribute to the molecular and clinical heterogeneity manifest in broken iMH lesions.

Considering significance for the treatment of MIH, it seems likely that attempts at remedial mineralization will benefit from prior removal of inhibitory proteins (Robinson *et al*., 1990; Mathu-Muju and Wright, 2006). Our results not only identify the major proteins needing removal, but also show that this need varies markedly, depending on integrity of the lesion surface. Additionally, these identified proteins have potential utility as biomarkers for characterizing MIH lesions clinically. The possibility of a causal link with leaked albumin resonates with the presumed multifactorial cause and sporadic occurrence of MIH. While speculative, this etiological possibility introduces key research questions about the cause of MIH that could ultimately unlock a path to its prevention.

ACKNOWLEDGMENTS

We thank Roger Hall and Garry Nervo for critique of the manuscript, and Paul O'Donnell (Bio21 Institute) for mass spectrometry support. This study was funded by the Melbourne Research Unit for Facial Disorders and NHMRC Australia.

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J Dent Res 89(10):1160-1165, 2010

APPENDIX

Appendix Table. Proteins Identified in iMH Enamel with Intact Surface (specimens 1–6) and with Post-eruptive Breakdown (specimens 7–11)

Name (UniProt acc.)	Body Fluid Localization[a]	Mass (kDa)		Specimens Identified	Peptides (n)	Coverage (%)	MASCOT Score
		Observed	Theoretical				
Serum albumin (P02768)	Serum, saliva, GCF	70	69	1	16	21	426
		60		3	2	4	89
				4	15	26	471
				5	10	21	197
				6	9	15	389
				3	3	8	87
				5	7	18	323
				6	12	15	389
Complement C3 beta chain (P01024)	Serum	70	71	7	2	1	165
Alpha-1-antitrypsin (P01009)	Serum, saliva, GCF	40	44	7	14	33	308
		25–30		7	3	16	122
Protein S100-A9 (P06702)	Saliva, GCF	25–30	13	7	3	24	54
		13		7	5	37	212
		13		11	6	56	158
Lactotransferrin (P02788)	Saliva, GCF	70	78	11	2	4	155
Leukocyte elastase inhibitor (P30740)	Blood, saliva	40	43	7	4	9	172
Antithrombin-III (P01008)	Serum, saliva	40	53	7	2	4	121
Serum albumin (P02768)	Serum, saliva, GCF	70	69	10	7	14	212
		60		10	14	24	461
		40		10	2	5	47
		32		10	3	2	50
		10		7	5	8	136
Hemoglobin subunit alpha (P69905)	Blood, saliva	13	15	7	6	38	117
		13		11	5	23	115
Hemoglobin subunit beta (P68871)	Blood, saliva, GCF	13	16	7	12	63	374
		13		10	2	12	51
		13		11	11	63	207
Hemoglobin subunit delta (P02042)	Blood, saliva	13	16	7	8	40	190
		13		11	6	50	145
Prolactin-inducible protein (P12273)	Saliva	13	17	11	2	15	85
Alpha amylase 1 (P04745)	Saliva	60	57	8	2	7	131
Ig kappa chain V-III region SIE (P01620)	Blood, saliva	25–30	12	7	2	16	130
		25–30		8	2	16	67
		25–30		11	2	16	91
Ig alpha-2 chain C region (P01877)	Blood, saliva	60	37	10	3	7	109
Uncharacterized protein c60rf58 (Q6P5S2)	Saliva	32	38	10	4	14	68
Serpin B3 (P29508)	Blood, saliva	27	45	10	2	4	48

[a]Protein localization previously described for serum [Pieper R *et al.* (2003), *Proteomics* 3:1345-1364], blood [Haudek VJ *et al.* (2009), *J Proteome Res* 8:3834-3843], saliva [Denny P *et al.* (2008), *J Proteome Res* 7:1994-2006], and gingival crevicular fluid (GCF) [Kojima T *et al.* (2000), *J Dent Res* 79:740-747].