Protein content of molar–incisor hypomineralisation enamel

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1. Introduction

Molar–incisor hypomineralisation (MIH) is used to describe enamel hypomineralisation in 1st permanent molars that is usually accompanied by less severe defects in the incisors.1,39 The enamel defects are usually asymmetric with well demarcated white/opaque, yellow or brown discoloration affecting cuspal areas and sparing the cervical areas. In many cases the incisor enamel is affected but often minimally. Chawla et al.1 suggested that yellow–brown enamel defects are more severe than white–opaque defects. It is widely accepted that the molecular aetiology of MIH is not understood.50,41

Farah et al.2 reported increased laser-induced fluorescence (LF) from MIH enamel that may be due to organic fluorophores. LF readings correlate with the organic content3,4 rather than the degree of mineralisation of enamel.5

The organic content of MIH enamel has yet to be assessed. The protein content can be deduced indirectly from its elemental composition. Jälevik et al.6 showed that MIH enamel had a higher carbon content than normal enamel. The high resistance of MIH enamel to acid etching is consistent with an increased organic content rather than carbonate substitution of the normal apatite lattice.7

The enamel protein content of sound enamel has been reported extensively.8–10 Several methods have been used to extract enamel protein.11–15 Comparison of trichloracetic (TCA) acid, acetic acid and urea led Porto et al.16 to recommend the use of 12% TCA for the extraction of protein from rat dental enamel at different stages of enamel development. For late maturation
stage enamel, TCA extracted 60% of the total protein content, whilst acetic acid or urea extracted less than 20%. TCA offers the combined advantage of dissolving the mineral phase of enamel and precipitating the extracted proteins.

The present study compares the relative amounts and nature of the protein content of sound and hypomineralised human enamel and identifies serum proteins in MIH enamel.

2. Materials and methods

2.1. Enamel samples

“Ethical approval to conduct the study was obtained from the New Zealand Multi-Region Ethics Committee (MEC/06/12/177, March 2007). Ten first permanent molars with different severities of MIH (and six mature molars with sound enamel) were collected following planned extractions from ten children of various ethnicities (age range: 7.5–10.5 years old). The reason for extraction varied from case to case, but was the result of a comprehensive management plan determined by a paediatric dentist and/or orthodontist. None of the teeth showed any sign of dental caries. Of the ten MIH molars, seven were randomly chosen and their mineral density measured using X-ray microtomography and was found to be reduced compared with normal enamel, consistent with enamel hypomineralisation.30 The MIH molars were divided into three groups (brown, yellow, opaque/white) based on Chawla et al.’s1 classification. The teeth were stored at 4 °C in distilled water with thymol crystals until they were tested.”

2.2. Protein extraction and quantitation

Protein was extracted from sound and MIH enamel using a modification of the method of Porto et al.16 A pilot study, which showed that 20% TCA was more effective at dissolving MIH enamel than 12% TCA, also allowed estimation of the amount of enamel needed to detect and characterize the precipitated protein. These amounts were: sound enamel 0.02–0.03 g; opaque or yellow enamel ~0.015 g; brown enamel ~0.008 g. One millilitre of 20% TCA was needed to dissolve 0.01 g of sound enamel or 0.005 g of opaque, yellow or brown enamel.

A sterile high-speed handpiece and bur under sterile saline irrigation was used to cut small pieces of enamel (about 0.25–0.50 mm in dimensions) from the teeth. Enamel pieces were collected from three sound teeth, three MIH teeth with yellow discoloration, and one MIH tooth with brown discoloration. The enamel pieces were crushed into coarse powder and weighed. Samples (Group A) of crushed sound enamel (0.0329 g) and crushed yellow enamel (0.0179 g) were dispersed in three equal parts in Eppendorf tubes whilst the crushed brown enamel (0.00814 g) was dispersed in two equal parts. To confirm these results, a separate group of enamel samples (Group B) was collected from sound and MIH enamel and crushed as described above. These enamel pieces were from three sound teeth (0.0326 g), two MIH teeth with white/opaque discoloration in the enamel (0.0118 g), three MIH teeth with yellow discoloration (0.0149 g), and one MIH tooth with brown discoloration (0.079 g).

TCA (990 µl of 20%) and phenylmethylsulphonylfluoride (PMSF, 10 µl of 100 mM in isopropanol) were added to each Eppendorf tube and the mixture incubated at room temperature. Sound enamel dissolved overnight, whilst MIH samples required 2 days. The samples were centrifuged (4 °C, 13,200 rpm, 10 min) and pellets for each group of enamel pooled after dissolution in 50 µl of 1% SDS. The protein content of each sample was estimated using a miniaturized version of the method of Lowry et al.17

2.3. SDS-polyacrylamide gel electrophoresis and mass spectrometry of tryptic fingerprints

Samples (10 µg) of the solubilised protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% polyacrylamide gel at 100 V for 90 min,18 stained with Coomassie Blue R250, and relative protein molecular weights were determined by comparison with molecular weight markers (PageRuler™ Unstained Protein Ladder, Fermentas Inc., MD, USA).

Excised segments of the Coomassie Brilliant Blue-stained gel were digested with trypsin (Sequencing Grade Modified Trypsin, Promega, Madison) and the profiles of tryptic fragments identified using MS/MS (LTQ-Orbitrap XL hybrid mass spectrometer, Thermo Scientific, San Jose, CA). For protein identification MS/MS data was searched against the UniProt/SWISS-PROT amino acid sequence database using the Mascot search engine (http://www.matrixscience.com). The identifications were filtered with a significance threshold of p < 0.05 and ions score cut-off of 35. The number of significant peptide identifications (and the corresponding sequence coverage) was then extracted for the identified proteins. Proteins with a minimum of two matches on the database were reported.

3. Results

The amounts of enamel protein detected using the Lowry method and their percentages by weight of enamel are shown in Table 1.

Electrophoretic profiles of proteins extracted from the different types of enamel are shown in Fig. 1. Strongly stained bands which migrated just ahead the 70 kDa protein standard were seen in the yellow and brown enamel samples from both groups, whilst background staining was seen in the sound or white/opaque enamel samples. There were also fainter bands in the yellow and brown MIH enamel samples with relative mobilities between the 70 kDa and 50 kDa protein standards. Tryptic fingerprint/mass spectrometry was performed on excised 50–70 kDa areas from all the lanes, even if no obvious stained bands were seen. Proteins common to all lanes were serum albumin, serum alpha-1-antitrypsin, and type I collagen (Table 2). Antithrombin III was present in yellow and brown enamel only, whilst ameloblastin was unique to brown enamel. Cytoskeletal keratins were also identified, but they are an expected contamination in MS analysis.19 Brown and yellow enamel lanes showed more abundant serum albumin and alpha-1-antitrypsin as evidenced by band intensity and the frequencies of database matches. For example, the
peptides identified from the mass spectrometry data (Fig. 2) showed significantly enhanced detection of protein fragments from serum albumin and alpha-1-antitrypsin in the brown and yellow enamel compared with sound and white/opaque enamel.

4. Discussion

The enamel pieces cut from sound enamel needed considerable pressure to obtain a coarse powder. Yellow and brown MIH enamel required much more modest pressure, implying these defective enamels will perform poorly in the oral cavity. Mahoney et al.20 showed that the hardness and elastic modulus of defective areas from MIH teeth were reduced by up to 80% compared with normal enamel, and suggested reduced mineralisation and elevated protein content might be responsible. Mahoney7 also observed that the classic etching patterns obtained with sound enamel were absent in MIH enamel. It was suggested that an increased protein content of MIH enamel might limit access of acid to the hydroxyapatite crystallites. The longer time required for MIH enamel dissolution in acid observed in the present study may also be due to its elevated protein content.

MIH enamel showed 8–21-fold higher protein content than sound enamel. Brown enamel had the highest protein content (15–21-fold greater), whilst the protein content of white/opaque and yellow enamel were both markedly higher (~8-fold greater) than sound enamel (Table 1). Other enamel defects such as the hypomaturation and hypocalcification types of amelogenesis imperfecta (AI) show similar, up to 20-fold, increases in organic content over sound enamel.21

SDS-PAGE separated bands of around 70 kDa were readily detected in yellow and brown MIH enamel only. Mass spectrometry of this region of the gel identified serum albumin, alpha-1-antitrypsin, and type I collagen in all enamel groups. The yellow and brown enamel showed the greatest abundance of serum albumin and alpha-1-antitrypsin as well as the presence of antithrombin III (Table 2 and Fig. 2). The intrinsic enamel protein ameloblastin was also identified, in the brown enamel sample only, but at the limits of detection (2 significant peptide hits).

The presence of type I collagen in sound and MIH enamel samples is unlikely to be contamination from dentine. Minute amounts of collagen were previously detected in dental enamel22–24 and Lin et al.25 observed that collagen fibrils from the dentine–enamel junction penetrate into the enamel. Eastoe8 also found the collagen component hydroxyproline in mature human enamel and concluded that small amounts of collagen were present. A recent study26 detected the two non-reducible cross-links of mature collagen and hydroxyproline in human dental enamel but at much lower concentrations than in dentine.

The number of peptides identified in protein samples by MS has been shown to accurately reflect the relative abundance of the proteins in a mixture.27,28 This is most clearly demonstrated for albumin in normal, white/opaque, yellow and brown enamel. The detection in white/opaque and sound enamel of serum albumin at a low level in the SDS-PAGE 50–70 kDa region required sensitive MS/MS analysis. In contrast, the numbers of database matches detected for serum albumin were 3–7-fold higher for yellow and brown enamel than for sound and white/opaque enamel (Table 2 and Fig. 2). Similarly, antitrypsin appeared at least 2-fold more abundant in yellow and brown enamel. Finally, antithrombin III was detected in brown and yellow enamel but not in white or opaque enamel. The increased relative abundances of serum albumin, alpha-1-antitrypsin and antithrombin III in yellow

### Table 1 – Protein amounts and percentages from different types of enamel.

<table>
<thead>
<tr>
<th>Group</th>
<th>Enamel type</th>
<th>Enamel pieces weight (g)</th>
<th>Total protein recovered (μg)</th>
<th>Percentage of protein in enamel (wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sound</td>
<td>0.0329</td>
<td>11.3</td>
<td>0.030%</td>
</tr>
<tr>
<td></td>
<td>MIH: yellow</td>
<td>0.0179</td>
<td>15.8</td>
<td>0.088%</td>
</tr>
<tr>
<td></td>
<td>MIH: brown</td>
<td>0.00814</td>
<td>38.09</td>
<td>0.46%</td>
</tr>
<tr>
<td>B</td>
<td>Sound</td>
<td>0.0326</td>
<td>10.44</td>
<td>0.032%</td>
</tr>
<tr>
<td></td>
<td>MIH: white/opaque</td>
<td>0.0118</td>
<td>33.97</td>
<td>0.28%</td>
</tr>
<tr>
<td></td>
<td>MIH: yellow</td>
<td>0.0149</td>
<td>38.16</td>
<td>0.25%</td>
</tr>
<tr>
<td></td>
<td>MIH: brown</td>
<td>0.0079</td>
<td>50.56</td>
<td>0.64%</td>
</tr>
</tbody>
</table>

Fig. 1 – Coomassie Blue staining of SDS-PAGE separated enamel proteins. Lane 1: marker, Lane 2: sound enamel (Group A), Lane 3: yellow MIH enamel (Group A), Lane 4: brown MIH enamel (Group A), Lane 5: sound enamel (Group B), Lane 6: opaque MIH enamel (Group B), Lane 7: yellow MIH enamel (Group B), Lane 8: brown MIH enamel (Group B).
and brown enamel samples only indicate the presence of serum-derived proteins in these MIH enamels.

Trace amounts of serum albumin have previously been found in both developing and mature enamel. Robinson et al.29 used SDS-PAGE and Western blots to monitor albumin content during the stages of enamel development in rat incisors. Intact albumin was detected during the secretion and transition stages, with a peak around the late secretion/early transition stage. Some degradation products of albumin were also found in the transition/early maturation stage, and very little albumin was detected in the late maturation stage. It is thought that albumin degradation begins during the transition stage and is almost complete by the maturation stage, leaving only trace amounts of the protein in the mature enamel.

X-ray microtomography shows that MIH enamel has a reduced mineral content.30 Might an over-abundance of albumin interfere with the mineralisation process? Studies of the interaction between enamel crystals and serum albumin support this suggestion. Menanteau31 demonstrated that albumin binds to apatite crystals in vitro. This binding appeared to inhibit the growth of both synthetic32 and natural enamel apatite crystals.33 Robinson et al.10 noted that albumin was mineral-bound in the transition and maturation stages, when the bulk of the matrix was removed, but not in the secretion stage. They suggested that solid state amelogenin may prevent albumin from reaching the crystals during the secretion stage. In the late transition and early maturation stage, access to the apatite crystals occurs when the amelogenin-rich matrix is degraded and removed. Serum albumin may also be degraded at this later stage, since both the intact 67 kDa protein and its degradation products were found in the transition stage. These findings suggest that albumin degradation may be a prerequi-site for maximal crystal growth in the maturation stage.

Related to this may be the finding that yellow and brown MIH enamel also contained the serine proteinase inhibitors (serpins) alpha-1-antitrypsin (52 kDa) and antithrombin (58 kDa). They can inhibit the enamel serine protease kallikrein 4 (KLK4) which is secreted into the enamel by ameloblasts.34 KLK4 secreted during the transition and maturation stages of amelogenesis degrades the organic matrix remaining from the secretion stage. This facilitates the continued deposition of minerals into enamel required for full mineralisation of hard enamel. Mutations in KLK4 cause poor enamel matrix degradation and are expressed clinically as hypomaturation A1.35 Antitrypsin and antithrombin impairment of KLK4 activity could result in enamel with elevated protein content and reduced mineral content. Combined with the effect of excess serum albumin, this may inhibit mineralisation and cause the severely compromised MIH enamel.

Table 2 – Proteins found in the different enamel groups. Only proteins with a minimum of 2 matches on the database are reported.

<table>
<thead>
<tr>
<th>Enamel type</th>
<th>Protein identified</th>
<th>Mascot score at a significance threshold of p &lt; 0.05</th>
<th>Number of significant peptide IDs</th>
<th>% sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sound</td>
<td>Human serum albumin</td>
<td>490</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 1 (I)</td>
<td>261</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 2 (I)</td>
<td>269</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Human alpha-1-antitrypsin</td>
<td>95</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>MIH: white/opaque</td>
<td>Human serum albumin</td>
<td>357</td>
<td>9</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 1 (I)</td>
<td>289</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 2 (I)</td>
<td>376</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Human alpha-1-antitrypsin</td>
<td>96</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>MIH: yellow</td>
<td>Human serum albumin</td>
<td>1752</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 1 (I)</td>
<td>108</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 2 (I)</td>
<td>52</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Human alpha-1-antitrypsin</td>
<td>93</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Human antithrombin III</td>
<td>84</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>MIH: brown</td>
<td>Human serum albumin</td>
<td>1343</td>
<td>53</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 1 (I)</td>
<td>316</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Human collagen alpha 2 (I)</td>
<td>256</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Human alpha-1-antitrypsin</td>
<td>174</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Human ameloblastin</td>
<td>94</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Human antithrombin III</td>
<td>71</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 2 – Number of peptides identified for individual proteins in each type of enamel.
This is the first report of the detection of alpha-1-antitrypsin and antithrombin III in dental enamel. It is not known how these proteins and serum albumin reach developing enamel. It seems likely that the source of serum albumin in developing enamel is external to the ameloblasts. Two studies have reported that mouse molar ameloblasts do not express albumin,\textsuperscript{36,37} implying that the albumin leaks through the ameloblasts layer into the developing enamel. It may be difficult to remove excessive amounts of albumin, especially in the presence of a serpin. Tarjan et al.\textsuperscript{38} physically insulted the developing incisor tooth germ in 4-day-old rats by using controlled trauma to the inferior border of the mandible. Haemorrhage surrounded the enamel organ in the traumatized area, with blood and labelled albumin penetrating between detached ameloblasts and entering the enamel. Robinson et al.\textsuperscript{29} showed that large amounts of albumin leaked into developing enamel at the late transition/early maturation stage but not in the secretory/early transition stage of post-mortem tissue. The vulnerability of late transition/early maturation stage to albumin influx was probably due to degeneration of capillaries and loss of ameloblast cell-to-cell contacts.

Robinson et al.\textsuperscript{10} suggested that amelogenin protection of apatite crystals from albumin in the secretion stage may have clinical consequences. Transition stage ameloblasts are most vulnerable; the vascularity is highest, the protective capacity of enamel is external to the ameloblasts. Two studies have reported that mouse molar ameloblasts do not express albumin,\textsuperscript{36,37} implying that the albumin leaks through the ameloblasts layer into the developing enamel. It may be difficult to remove excessive amounts of albumin, especially in the presence of a serpin. Tarjan et al.\textsuperscript{38} physically insulted the developing incisor tooth germ in 4-day-old rats by using controlled trauma to the inferior border of the mandible. Haemorrhage surrounded the enamel organ in the traumatized area, with blood and labelled albumin penetrating between detached ameloblasts and entering the enamel. Robinson et al.\textsuperscript{29} showed that large amounts of albumin leaked into developing enamel at the late transition/early maturation stage but not in the secretory/early transition stage of post-mortem tissue. The vulnerability of late transition/early maturation stage to albumin influx was probably due to degeneration of capillaries and loss of ameloblast cell-to-cell contacts.

Robinson et al.\textsuperscript{10} suggested that amelogenin protection of apatite crystals from albumin in the secretion stage may have clinical consequences. Transition stage ameloblasts are most vulnerable; the vascularity is highest, the protective capacity of amelogenin is declining rapidly, and the enamel is very porous. Hyperaemic insult caused by physical trauma could allow excessive albumin to enter developing enamel in the transition stage and impair final crystal growth. This would lead to the eruption of immature, porous hypomineralized enamel.

5. Conclusions

Serum proteins appear to become incorporated into dental enamel during its formation in MIH. The large amounts of albumin bound to apatite crystals may inhibit their growth. Serum proteinase inhibitors may prevent degradation of albumin (and other matrix proteins) and further impair enamel maturation. We suggest that the excessive amounts of albumin (and other serum proteins) detected in MIH enamel may be due to a traumatic injury to the enamel organ. The nature of the trauma, whether pathological or physical, requires further investigation.

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References