Supporting Information

Relationships between protein and mineral during enamel development in normal and genetically altered mice

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Materials and Methods (for data shown in Supporting Figures and Table)

Microweighing

All microweighings in this study were done using a Sartorius SC2 microbalance (Sartorius, Göttingen, Germany) and small 12 mm diameter aluminum dishes (Fisher Scientific Company, Ottawa, ON, CAN). Mineral samples of known types were obtained from Clarkson Chromatography Products Inc (South Williamsport, PA, USA), Sigma-Aldrich Canada (Oakville, ON, CAN), Monsanto (St. Louis, MO, USA), the National Institute of Standards and Technology (Gaithersburg, MD, USA) and the Forsyth Institute (Cambridge, MA, USA; courtesy of Dr. Henry C. Margolis). Small aliquots of each mineral were dispensed into the aluminum weighing dishes and placed in a drying oven at 45 °C for 48 h. After cooling to room temperature, each sample was weighed on the microbalance and placed in individual precleaned 15 × 15-mm Coors crucibles (1.3 mL capacity; Fisher Scientific). The crucibles were heated in a muffle furnace (Fisher Scientific) at 575 °C for 18 or 24 h depending upon experiment. The samples were cooled, and the material in the crucibles was transferred back to aluminum dishes and reweighed. This procedure was repeated at least twice for some samples and as many as 7 times for others. The percent original weight was computed as (after heating weight / before heating weight) x 100.

Assay of protein content

All procedures involving rats were reviewed and approved by the Downtown Campus Facility and University Animal Care Committees of McGill University. The procedures used for processing animals weighing ~100 g, obtaining sequential enamel strips from freeze dried incisors, weighing and ashing enamel strips at 575 °C, and for doing Lowry protein assays of

proteins extracted from crushed enamel strips were exactly as described previously (1,2) Standard protein curves covering a range of 0 – 100 μg were constructed by using the microbalance to determine a known starting weight of 1x crystallized and fatty acid free bovine serum albumin (BSA; Sigma-Aldrich) and affinity purified recombinant mouse amelogenin (rM179; made in house (3)), dissolving these powders in distilled water to a concentration of 2 mg/mL, then serially diluting these stocks in distilled water to the desired amounts per assay tube. Sets of sequential enamel strips from 7 mandibular rat incisors were removed and crushed, and proteins were extracted (2) and used to make curves for enamel as read against the BSA and rM179 standard curves. Other sets of sequential enamel strips from 11 mandibular rat incisors were used to construct a volatiles curve for enamel.

References

1. SMITH CE, CHONG DL, BARTLETT JD, MARGOLIS HC. Mineral acquisition rates in developing enamel on maxillary and mandibular incisors of rats and mice: Implications to extracellular acid loading as apatite crystals mature. *J Bone Miner Res* 2005; **20**: 240-249.

2. MCKEE MD, WEDLICH L, POMPURA JR, NANCI A, SMITH CE, WARSHAWSKY H. Demonstration by staining and radioautography of cyclical distributions of protein at the enamel surface in rat incisors. *Arch Oral Biol* 1988; **33**: 413-423.

3. SIMMER JP, LAU EC, HU JC, AOBA T, LACEY M, NELSON D, ZEICHNER-DAVID M, SNEAD ML, SLAVKIN HC, FINCHAM AG. Isolation and characterization of a mouse amelogenin expressed in *Escherichia coli*. *Calcif Tissue Int* 1994; **54**: 312-319.

Table S1: Weight Changes Following Heating of Mineral Samples at 575°C

Mineral Sample[1]	Type	Source	Heating Time	Percent Original Weight			N[4]
				Mean	±	SD	
OHAp	ceramic grade	Clarkson	24 h	99.96	±	0.34	4
OHAp	12.4 m^2/g [2]	Forsyth Institute	18 h	97.84	±	0.25	4
OHAp	undefined	Forsyth Institute	18 h	97.28	±	0.33	5
OHAp	18.4 m^2/g	NIST Standard[3]	18 h	97.11	±	1.23	7
$CaCO_3$	99% anhydrous	Sigma-Aldrich	18 h	96.09	±	1.12	6
OHAp	undefined	Sigma-Aldrich	18 h	95.16	±	0.01	2
OHAp	59.6 m^2/g	Monsanto	18 h	95.05	±	0.25	5
$CaCO_3$	99% anhydrous	Sigma-Aldrich	24 h	90.86	±	1.53	2
OCP	undefined	Clarkson	18 h	90.66	±	0.16	5
OCP	undefined	Clarkson	24 h	89.64	±	0.12	3

[1] Abbreviations: OHAp, calcium hydroxyapatite; $CaCO_3$, calcium carbonate; OCP, octacalcium phosphate; all samples were initially placed in a drying oven at 45°C for 48 h and cooled before obtaining original starting weighing

[2]This is the specific surface area of the crystalline preparation; smaller numbers imply larger crystal sizes

[3]NIST calcium hydroxyapatite standard (2910) is fully characterized and contains by weight 1.59% bound water, 0.592% HPO4 (acid phosphate) and 0.032% CO_3 (carbonate). The expected loss in weight resulting from evaporation of these components during heating at 575°C is therefore 2.214% (observed was 2.89%)

[4]Number of independent repeat measurements for obtaining estimate of mean

Table S2: Relationships Between Mineral and Volatiles During Enamel Development

	A Greatest Amount of Volatiles Detected (μg)				B Mineral Content in This Enamel Strip (μg)				C Volatile Content in Strip With Highest Mineral Content (μg)				D Mineral Content in This Enamel Strip (μg)				E Lowest Volatile Content Detected in Maturation (μg)			
Maxillary Incisors																				
Genotype	Mean	±	SD[1]	Strip #	Mean	±	SD	%[2]	Mean	±	SD	Strip #	Mean	±	SD	%	Mean	±	SD	Strip #
Enam$^{+/+}$	21.3	±	1.6	2	18.7	±	4.2	47	11.5	±	2.6	3	60.8	±	8.1	84	11.5	±	2.6	3
Enam$^{+/-}$	20.6	±	2.2	2	23.2	±	9.6	53	14.3	±	2.6*	3	62.6	±	11.3	81	14.3	±	2.6*	3
Enam$^{-/-}$	6.1	±	2.3*†	4	8.4	±	3.7*†	58	5.7	±	1.4*†	5	10.9	±	3.6*	66	5.1	±	1.3*†	6
Ambn$^{+/+}$	24.8	±	4.0	2	17.1	±	3.4	41	15.1	±	4.0	3	53.1	±	7.0	78	15.1	±	4.0	3
Ambn$^{+/-5,6}$	19.7	±	1.7*	2	17.6	±	3.3	47	17.2	±	2.6	4	77.2	±	14.2*	82	10.1	±	2.8*	3
Ambn$^{-5,6/-5,6}$	6.0	±	1.6*†	4	10.6	±	1.8*†	64	5.6	±	1.0*†	6	10.9	±	1.8*†	66	5.5	±	1.4*†	5
Mmp20$^{+/+}$	22.6	±	3.0	2	19.1	±	5.3	46	13.5	±	4.0	3	54.9	±	13.8	80	13.5	±	4.0	3
Mmp20$^{+/-}$	24.2	±	2.1	2	19.8	±	5.4	45	13.2	±	3.7	3	53.8	±	9.2	80	13.2	±	3.7	3
Mmp20$^{-/-}$	14.0	±	2.9*†	3	26.2	±	4.9*†	65	12.9	±	3.8	6	32.2	±	4.7*†	71	12.2	±	2.1	5
Klk4$^{+/+}$	23.8	±	2.6	2	13.7	±	2.5	37	14.6	±	3.1	4	75.2	±	12.2	84	14.6	±	3.1	4
Klk4$^{+/-}$	24.0	±	1.4	2	17.7	±	5.4*	42	15.6	±	3.0	4	78.1	±	10.7	83	15.5	±	2.5	3
Klk4$^{-/-}$	23.3	±	1.5	2	20.7	±	4.8*	47	18.8	±	2.2*†	4	76.2	±	6.5	80	15.8	±	2.9	3
Mandibular Incisors																				
Genotype	Mean	±	SD	Strip #	Mean	±	SD	%	Mean	±	SD	Strip #	Mean	±	SD	%	Mean	±	SD	Strip #
Enam$^{+/+}$	26.2	±	4.9	2/3[3]	31.0	±	9.3	51	15.8	±	5.2	5	119.1	±	24.3	88	10.8	±	2.3	4
Enam$^{+/-}$	22.2	±	3.2*	3	22.2	±	3.4*	50	11.3	±	3.1*	9	61.0	±	10.2*	84	11.3	±	3.1	9
Enam$^{-/-}$	9.7	±	5.0*†	5	16.7	±	8.6*	63	8.8	±	2.9*	7	17.9	±	5.2*†	67	7.5	±	4.2*†	9
Ambn$^{+/+}$	28.5	±	5.0	3	42.5	±	13.0	60	18.5	±	5.6	5	133.5	±	21.0	88	14.5	±	3.5	4
Ambn$^{+/-5,6}$	27.2	±	2.7*	3	29.7	±	5.8*	52	14.6	±	2.6*	6	102.9	±	11.8*	88	12.6	±	4.2	5
Ambn$^{-5,6/-5,6}$	6.2	±	2.0*†	4	11.7	±	2.5*†	65	5.6	±	2.7*†	8	12.8	±	5.8*†	69	4.4	±	1.5*†	6
Mmp20$^{+/+}$	30.0	±	3.5	3	30.9	±	4.5	51	12.9	±	5.4	5	101.2	±	7.7	89	12.9	±	5.4	5
Mmp20$^{+/-}$	30.2	±	1.3	3	30.5	±	5.8	50	18.4	±	5.7*	5	105.5	±	7.2	85	16.9	±	2.5*	4
Mmp20$^{-/-}$	15.0	±	2.1*†	4	31.0	±	7.1	67	13.0	±	4.1 †	8	41.8	±	4.3*†	76	13.0	±	4.1 †	8
Klk4$^{+/+}$	32.5	±	2.2	3	31.9	±	4.1	50	12.2	±	2.4	5	108.0	±	8.2	90	12.2	±	2.4	5
Klk4$^{+/-}$	30.5	±	4.0	3	39.1	±	9.9*	56	18.2	±	3.9*	6	113.4	±	10.8	86	16.4	±	3.3*	5
Klk4$^{-/-}$	31.6	±	2.1	3	33.0	±	6.0	51	24.7	±	2.6*†	6	108.8	±	6.9	81	20.8	±	1.6*†	5

[1] SD = standard deviation; * indicates mean is significantly different ($p < .05$) from wild type (+/+); † indicates mean is significantly different ($p < .05$) from heterozygous (+/-)

[2]% = percent mineral by weight; the number of observations per genotype are too low to detect differences in means by the Fisher Exact Test (used when numbers represent ratios)

[3]Values shown are the average of strips 2 and 3 for this genotype (due of misaligned strip dissections)

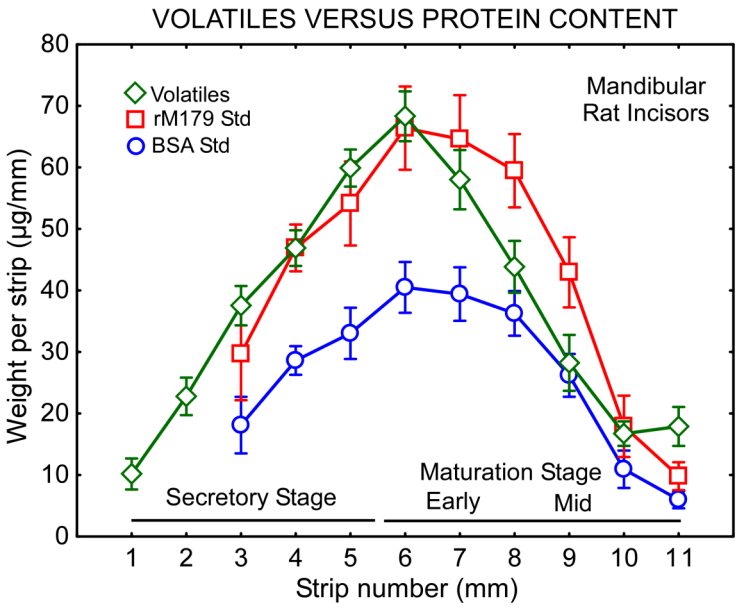

Figure S1. Graph showing estimates of the amount of protein present in 1-mm-long strips of enamel removed sequentially from mandibular incisors of 100 g rats; means ± 95% confidence intervals (there are twice as many enamel strips per length in rats compared to mice). The blue line and symbols show Lowry estimates of the amount of proteins present in enamel strips based on a standard curve made from BSA whereas the red line and symbols show estimates of protein content based on rM179 as standard. The green line and symbols show estimates of the amount of volatiles present in the same strips based on direct measurements of strip weight before and after heating the strips at 575°C for 18 h (ashing). ***Key:*** BSA, bovine serum albumin; rM179, recombinant mouse amelogenin.