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Biophysical characterization of synthetic amelogenin C-terminal peptides

Feroz Khan^a, Wu Li^b, and Stefan Habelitz^{a,*}

^aDepartment of Preventive and Restorative Dental Sciences, University of California, Parnassus Avenue 707, San Francisco, CA 94143, USA

^bDepartment of Oral and Craniofacial Sciences, University of California, San Francisco, USA

Abstract

Amelogenin plays a key role in the formation of the highly mineralized structure of tooth enamel. During secretory stage, amelogenin is cleaved gradually by a protease, MMP-20 (matrix metalloproteinase-20), releasing hydrophilic C-terminal peptides. In this study, synthetic C-terminal peptides (28, 17 and 11 residues), mimicking the native peptides are explored *in vitro* for their biophysical characterization. A sudden drop in ζ -potential upon addition of calcium or phosphates was observed, which was also accompanied by an increased aggregation propensity of the peptides. In most of the conditions, particle size increased at a pH 2–3 units higher from pI of the peptides, while the peptides existed as smaller particles (<2 nm) near their pIs and in the acidic range. They showed poor affinity for calcium and phosphates comparable to full-length amelogenin and variants. The secondary structure determination showed that the 11 amino acid peptide contained defined secondary structure comprising beta-sheets and turns. AFM analysis revealed the presence of thin disk-like nanostructures of a diameter of 54.4 nm for 28 aa peptide and 54.9 nm for 11 aa, whereas no definite structures were observed for 17 aa peptide. It is concluded that the amelogenin C-terminal peptides are capable to interact with calcium and phosphate ions, self assemble into nanostructures, and may have some secondary structures, hence, they may have some role in enamel synthesis.

Keywords

enamel; ameloblast; tooth development; enamelysin; biomineralization

Enamel formation is one of the most interesting biological phenomena, that involves a series of complex cellular and molecular events occurring in the matrix of a growing tooth (1). These events involve protein secretion (2), protein self-assembly (3), protein-mineral interactions (4) and finally proteolysis (5) leading to formation of highly mineralized enamel (6). In the process of mineralization successive incorporation of inorganic minerals, in the matrix, results in the hardest tissue in the human body achieving toughness values similar to the collagen-based mineralized tissues bone and dentin (7).

Amelogenin being the major protein constitutes 90% of the total protein found in the extracellular matrix in a developing dental enamel, the remaining 10% includes-ameloblastin, enamelin, amelotin and proteases (1,8). The property of amelogenins to self-assemble into nanospheres and nanochains lead to the common notion that they may have a

Stefan Habelitz, Department of Preventive and Restorative Dental Sciences, University of California, Parnassus Avenue 707, San Francisco, CA 94143, USA Telefax: +1-415-4760858. Stefan.habelitz@ucsf.edu.

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potential role in scaffolding proteins to guide nucleation and uniaxial enamel crystal growth (9,10). Amelogenin null mice exhibited dental hypoplasia with complete loss of enamel rod structure and reduced thickness of enamel (11). Mutations in the human *AMELX P5* gene lead to amelogenesis imperfecta, a hereditary disease of enamel malformation (12). The full-length amelogenin is mainly composed of hydrophobic residues and a relatively much shorter, one tenth of the length, hydrophilic C-terminus (13). The amino acid sequences of amelogenin are highly conserved and in particular the terminal sequences are almost identical among several mammalian species (14).

During early tooth development, a zinc and calcium dependent proteolytic enzyme matrix metalloproteinase-20 (MMP-20) is secreted by the ameloblasts and participates in the stepwise processing of enamel matrix proteins while crystal growth of nanometer thin apatite ribbons occurs simultaneously (15). Only during the final steps of enamel mineralization, the organic matrix is further hydrolyzed and almost completely removed by the proteolytic activity of another protease, KLK-4, which is expressed during the maturation stage of amelogenesis (16,17).

MMP-20 has specific cleavage sites on enamel matrix proteins and cleaves the full-length human amelogenin H175 to give tyrosine-rich amelogenin polypeptide (TRAP), the 23 kDa amelogenin peptide (H164), the 20 kDa peptide (H147) and various C-terminal fragments (5,18,19). The charged C-terminus of amelogenin has significant apatite binding affinity suggesting that it is able to block certain crystallographic planes and thus able to control morphology of the crystals (20). Some other studies have indicated that removal of the C-terminal hydrophilic residue of amelogenin does not affect its ability to inhibit crystal growth of calcium phosphates in directions perpendicular to the *c*-axis (21,22). It was also found that crystals that formed in the presence of the hydrophilic C-terminus were organized into parallel arrays, whereas crystals forming in the absence of the hydrophilic C-terminus under the same condition were randomly arranged (21). It led to the conclusion that the hydrophobic N-terminal region of amelogenin inhibits crystal nucleation and growth, while the C-terminal domain plays a role in the parallel alignment of crystals within an enamel rod (21). On the other hand, it has also been demonstrated that the loss of the charged C-terminus during proteolytic processing of amelogenin results in reduced affinity to hydroxyapatite and a reduction in ability to inhibit crystal growth (20,23). Solid state NMR studies have indicated that the C-terminus preferentially orients itself next to the hydroxyapatite (HAP) surface (24). The C-terminus in LRAP, a naturally occurring splice variant of full length amelogenin with preserved N- and C-termini, lacks any definite three dimensional folding and shows random coil structures with significant mobility (25,26). The binding studies of rLRAP and full length human amelogenin (rH174) with calcium have been carried out using isothermal titration microcalorimetry (ITC). Amelogenin (rH174) showed affinity constant (K_a) 1332 M^{-1} for calcium, whereas rLRAP showed slightly higher affinity constant ($K_a = 8551 \text{ M}^{-1}$) (4). All these observations provided enough evidence that the C-terminus is important in the interaction of amelogenin with the calcium phosphate crystals; and may have a role in directing mineralization.

Proteins and peptides behave like charged colloidal particles in aqueous solutions due to charged side chains of certain amino acids. Interaction of proteins with various ions can change their surface charge. ζ -potential is an indirect measure of the surface charge of the particles in colloidal systems. It can be defined as, the electric potential present at the boundary of the slipping or shear plane of a particle moving through a neutral dispersion medium (27,28). The ζ -potential measurements were carried out for full length amelogenin (rH174) and its MMP-20 cleavage products (rH163 and rH146). These observations indicated that aggregation of these amelogenins occurred when ζ -potentials were minimal (29).

In this study, biophysical characterization of the C-terminal peptides is carried out and compared with recombinant human full length amelogenin (rH174) and N-terminal variants (rH163 and rH146). These *in vitro* studies test if the mineral ion binding activity of hydrolyzed fragments of amelogenins differ significantly thus indicating a possible role of C-terminus during enamel synthesis.

Material and Methods

The three C-terminal peptides M28D (MQPLPPMLPD LTLEAWPSTD KTKREEVD), M17P (MQPLPPMLPD LTLEAWP), S11D (STDKTKREEVD) (Fig. 1) were commercially synthesized by CS Bio (Menlo Park, CA, USA) with a purity of 97–99% (based on supplier's HPLC and MS data). The amelogenins (rH174, rH163 and rH146) were expressed in BL21DE3 plyS E. coli and purified on C4 hydrophobic beads (30). The purity of the protein batches was above 95% as assessed by HPLC, SDS-PAGE and MS (data not shown).

Particle Size and ζ -potential measurements

Particle sizes of peptides were measured by dynamic light scattering using a Zetasizer Nano (Malvern, UK) operating at a measurement range of 0.6 nm to 3 μ m. The peptides (1 mg/ml for particle size and 0.1 mg/ml for ζ -potential) were dissolved in water, exploiting their intrinsic buffering property, desired pHs were obtained by adding 0.1 M HCl/KOH; and effect of pH on particle size and ζ -potential was recorded (Fig. 2). To explore the effect of mineral ions (Fig. 3), the peptides were dissolved at 1 mg/ml concentration in 10 mM acetate (pH 3–5) or 10 mM Tris-HCl (pH 7) buffer containing 50 mM KCl. Aliquots of CaCl_2 or KH_2PO_4 from 1 M stock solutions were added to achieve final concentration as 0.1, 0.25, 0.5, 1, 5, 7.5 and 10 mM. When required, pH was adjusted with 0.1 M HCl/KOH. The reported particle sizes correspond to the hydrodynamic diameters derived from the time correlation function of the particle number density. The ζ -potential was measured by dispersing 0.1 mg/ml protein in 10 mM acetate (pH 3–5) or 10 mM Tris-HCl (pH 7) buffer containing 50 mM KCl, in a dip-cell cuvette using the Zetasizer Nano (Malvern, UK). An alternating voltage of 20 V was applied, inducing movement of protein molecules and aggregates as a function of their surface charges. Each data point was the result of averaging over 100 acquisitions, each lasting about 10 s. The final values reported here are the statistical averages of three independent measurements.

Fluorescence measurement and mineral ion binding data analysis

Fluorescence measurement were carried out using a fluorescence multi-well plate reader (Genesys V; Thermo-Scientific, Waltham, MA, USA). The peptides (1 mg/ml) and amelogenins (0.25 mg/ml) sample in 10 mM acetate (pH 3–5) or 10 mM Tris-HCl (pH 7.0–8.4) buffer containing 50 mM KCl were placed in 96-well black plates at 25 °C. Aliquots of CaCl_2 or KH_2PO_4 from 0.1 or 1 M stock solutions were added to achieve final concentration as 0.1, 0.25, 0.5, 1, 5, 7.5 and 10 mM. When required, pH was adjusted by 0.1 M HCl/KOH. Samples were excited at 280 (rH174, rH163, rH146, M17P) or 295 (M28D) nm and emission spectra were recorded for wavelength ranging from 300 to 400 nm. Each spectrum was an average of five accumulations. The fluorescence maxima for all samples were considered for further analysis. Corrections were made to compensate the dilution effect upon addition of mineral ion solution to peptide/protein samples. All represented datasets are the average of three independent sets of experiments with SD less than 5%.

The association constants (K_a) were calculated according to the method by CHIPMAN et al. (31). The abscissa intercept of the plot of $\log [C]_f$ against $\log \{(\Delta F)/(F_c - F_\infty)\}$, where $[C]_f$ is

the free ligand concentration (Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$) not bound to peptide (protein), yielded pK_a value for the ion binding reaction to the peptide according to the relationship

$$\log [(F_0 - F_c)/(F_c - F_\infty)] = \log K_a + \log \{[C]_t - [P]_t(\Delta F/\Delta F_\infty)\}$$

where, F_0 is the initial fluorescence intensity, F_c is the fluorescence intensity of the peptide at any point during the titration, $[P]_t$ is the total protein concentration, ΔF_∞ is the change in fluorescence intensity at saturation binding, $[C]_t$ is the total ligand (Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$) concentration and $[C]_f$ is the free ligand concentration, given by,

$$[C]_f = [C]_t - [P]_t(\Delta F/\Delta F_\infty)$$

Circular dichroism spectroscopy

CD (circular dichroism) spectra were recorded on a computer interfaced Olis DSM-20CD spectropolarimeter using quartz cell of 2 mM path length. Spectra were recorded from 180–280 nm at protein concentrations 0.53 mM (M28D), 0.89 mM (M17P) and 1.3 mM (S11D) in 10 mM acetate (pH 3–5) or 10 mM Tris-HCl (pH 7.0) buffer containing 50 mM KCl. Aliquots of CaCl_2 or KH_2PO_4 from 1 M stock solutions were added to achieve desired final concentration. Each spectrum is an average of three accumulations and smoothened by adjacent averaging.

AFM Imaging

The solutions of the peptides (1 mg/ml) in 10 mM acetate (pH 3–5) or 10 mM Tris-HCl (pH 7.0) buffer containing 50 mM KCl were used to prepare AFM discs (10). The AFM disks for control images were prepared in the same way without addition of peptides (images not shown). The images were recorded on an atomic force microscope (AFM) (Nanoscope III; Digital Instruments, Santa Barbara, CA, USA) in dry conditions using tapping mode with a Si-tip ($r < 10$ nm, $l = 125$ μm) (Tap 300, Budgetsensors) at a resonance frequency of ~ 300 kHz. The particle sizes were measured from the AFM graph using Nanoscope software, and an average size was calculated from 10 randomly selected particles, and the statistical significance was determined by t-test.

Results

Particle size and ζ -potential

The effect of pH on particle size and ζ -potential for the all three peptides is shown in Fig. 2. The presence of external buffer ions affects ζ -potential, and it could lead to different ζ -potential in the presence of different buffers even at the same pH. Hence, to keep identical ionic condition, use of any salt and external buffering agent was avoided. These data were collected, by adjusting pH by HCl/KOH; exploiting, however poor, intrinsic buffering capacity of the peptide itself. ζ -potential dropped from more than +25 mV to less than –30 mV, when pH was increased from below 3 to above 9 for all three peptides. The pH at which ζ -potential equals zero was considered as experimental pI of the peptide which was within the range of their theoretical values based on titratable groups of the peptides (32). The experimental pI was 4.6 (theoretical 4.4) for M28D, 4.5 (theoretical 3.7) for M17P and 4.5 (theoretical 4.9) for S11D. The particle size was less than 2 nm for M28D and M17P in acidic pH. When pH was gradually increased by addition of KOH; these peptides tend to aggregate at pH 6 and above (Fig. 2).

The effect of Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$ was studied in the presence of buffers to achieve greater buffering capacity required due to increasing concentrations of calcium and phosphates ions (Fig. 3). However due to difference in the ionic composition the data presented in Fig. 2 and Fig. 3 shows certain differences. Addition of CaCl_2 up to 1 mM resulted in an initial drop of ζ -potential prominently at pH 3 and 7, which was followed by a gradual increase (Fig. 3). However, the effect was inconspicuous at pH 5. The particle size remained unchanged at pH 3 and 5 for M28D but increased at pH 7 with the addition of Ca^{2+} (Fig. 3A). On the other hand, M17P showed no effect of Ca^{2+} at pH 3, but at pH 5 and 7, an increase in the particle size was observed due to Ca^{2+} induced aggregation (Fig. 3C). The peptide S11D, which exists in solution as aggregated particles (200–300 nm), probably due to strong intermolecular ionic forces, increased moderately in particle size (500–600 nm) at pH 5 only (Fig. 3E).

Addition of KH_2PO_4 in M28D solution at pH 3 resulted in the opposite effect on ζ -potential compared to the one observed for CaCl_2 (Fig. 3B). ζ -potential increased initially for concentrations of KH_2PO_4 up to 1 mM and later decreased gradually. This indicates different mechanism for ion adsorption for this peptide at pH 3. The effect of $\text{H}_x\text{PO}_4^{x-3}$ at other pH was the same as for Ca^{2+} in spite of its negative charge. M17P and S11D also showed same pattern of ζ -potential change as observed for Ca^{2+} (Fig. 3D & 3F). The particle size of M28D was less than 2 nm at pH 3–5 and did not show any distinctive effect of $\text{H}_x\text{PO}_4^{x-3}$ but increased from 100 nm to 500 nm in a similar way as observed for Ca^{2+} (Fig. 3B). M17P did not show any effect of $\text{H}_x\text{PO}_4^{x-3}$, or Ca^{2+} , on particle size at pH 3. However, at pH 5 and 7 both Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$ showed much prominent increase in particle size compare to other peptides (Fig. 3D).

Fluorescence spectroscopy and mineral ion binding

The intrinsic fluorescence spectra of the peptides and amelogenins are given in Fig. 4. To overcome protein precipitation near pI , the binding studies were carried out at higher pH for rH174 (pH 7.4), rH163 (pH 8) rH146 (pH 8.4) compare to peptides (pH 7). The peptide S11D could not be analyzed since it does not contain any intrinsic fluorophore. The other two peptides M28D and M17P contain single tryptophan, which showed emission maxima (λ_{max}) at 358 and 360 nm, respectively. The tryptophan emission near 360 nm in both peptides indicates its sufficient accessibility to the solvent (water), and rules out possibility of formation of any hydrophobic core surrounding it. However M28D showed a sharper emission peak as compared to M17P (Fig. 4A & 4B), which indicates difference in the microenvironment of the tryptophan in the peptides in spite of identical flanking sequence. Hence the two peptides may have different conformations, where M17P seems to have a more irregular and heterogeneous conformations than M28D. The amelogenin rH174 showed different λ_{max} at different pHs (Fig. 4C), which indicated changes in the conformation of the rH174 associated with the pH change. Emission maxima at pH 7.4 is 342 nm which shifted to 352 nm at pH 3–5 suggesting a relatively less compact tertiary structure at acidic pHs, which results in increased solvent accessibility of tryptophan as previously reported (29). The two other variants rH163 and rH146 also showed similar trend (Fig. 4D-E). Decrease in emission maxima in rH163 (340 nm) and rH146 (338 nm) compare to rH174 (342 nm), indicates gradual removal of highly hydrophilic C-terminal leads to compactness of tertiary structure and formation of more hydrophobic core.

Fluorescence titration of the peptides and rH174 with CaCl_2 and KH_2PO_4 resulted in quenching of the tryptophan fluorescence without any shift in the λ_{max} (Fig. 5 insets). Fluorescence quenching is caused by changes in microenvironment of Trp, which may be associated with peptide aggregation/rearrangement induced by ion binding. The fluorescence intensity at the λ_{max} was used, at the given concentration of the Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$, to obtain equilibrium binding plots and determination of affinity constants (Fig.

5, Table 1) (representative plots are shown only for calcium at one pH, other pHs and plots for KH_2PO_4 are omitted for brevity). Affinity constants obtained for rH174 were comparable to that reported earlier using isothermal titration calorimetry (ITC) at pH 7.5 (4); in addition, binding with $\text{H}_x\text{PO}_4^{x-3}$ and at acidic pHs is also reported here. Both the peptides and rH174 showed poor binding to Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$ with affinity constants in the millimolar range. Amelogenin (rH174) showed increase in affinity constants for Ca^{2+} and decrease for $\text{H}_x\text{PO}_4^{x-3}$ with increasing pH (5 to 7), indicating a significant contribution of charged residues for the mineral ion interactions (Table 1). Whereas, extreme acidic pH (=3) significantly disturb the protein conformation, as observed in spectrofluorometry, leading to very weak binding for Ca^{2+} ($K_a = 822 \text{ M}^{-1}$) and almost no binding to $\text{H}_x\text{PO}_4^{x-3}$ (Table 1).

rH163 ($K_a = 3876 \text{ M}^{-1}$) showed more and rH146 ($K_a = 625 \text{ M}^{-1}$) less affinity for Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$ compare to rH174 (Table 1). The peptide M17P showed similar trend like rH174 for binding to Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$, the binding to Ca^{2+} increased but decreased with $\text{H}_x\text{PO}_4^{x-3}$ with the increasing pH (5 to 7). Extreme acidic pH (<3) also resulted in relatively weak binding to Ca^{2+} and no binding to $\text{H}_x\text{PO}_4^{x-3}$. On the other hand, M28D showed similar binding trends for Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$ at all pHs studied (Table 1). M28D and rH174 showed similar affinity trend for Ca^{2+} , as binding increased with increase of pH from 3 to 7/8.5. A comparison, near physiological pH (≈ 7), revealed that binding to Ca^{2+} is twice stronger than $\text{H}_x\text{PO}_4^{x-3}$ for rH174 ($K_a = 1538 \text{ M}^{-1}$ for Ca^{2+} and $K_a = 750 \text{ M}^{-1}$ for $\text{H}_x\text{PO}_4^{x-3}$) and M17P ($K_a = 1428 \text{ M}^{-1}$ for Ca^{2+} and $K_a = 596 \text{ M}^{-1}$ for $\text{H}_x\text{PO}_4^{x-3}$), whereas it is nearly equal for M28D ($K_a = 1132 \text{ M}^{-1}$ for Ca^{2+} and $K_a = 1314 \text{ M}^{-1}$ for $\text{H}_x\text{PO}_4^{x-3}$) (Table 1).

Secondary structures

Secondary structures of the peptides were studied using circular dichroism spectra in Far-UV region. The peptide M28D and M17P showed similar circular dichroism spectra in Far-UV region 190–250 nm. M28D and M17P CD spectra were missing major characteristic negative peaks related to α - or β -structures at 220 nm, and showing noisy or negatives peaks at 190 nm characterizing the random coiled structures (33,34) (Fig. 6A,B). However, unexpectedly, both peptides showed a prominent negative peak at $\sim 235 \text{ nm}$ with $-15,000 \text{ deg.cm}^2/\text{dmol}$ intensity (Fig. 6A,B). This type of the peak has not been reported for proteins but is characteristic of lactones, a cyclic ester, of sialic acid (35). At this stage, the origin of these peaks is not certain, possibly it is not a contaminant as purity of the peptides is established HPLC and MS data (data not shown). Change in pH or addition of 0.1–10 mM of CaCl_2 or KH_2PO_4 at pH 3, 5 or 7 (data shown only for pH5 at 5 mM Ca^{2+} and $\text{H}_x\text{PO}_4^{x-3}$) did not cause any significant change in CD spectra (Fig. 6A,B).

The peptide S11D, unlike the other two peptides, was characterized by a strong negative peak at $\sim 220 \text{ nm}$ with $-27,000 \text{ deg.cm}^2/\text{dmol}$ intensity and small positive peaks at 200 nm (Fig. 6C), characteristic of a folded protein (33;34). The spectra were recorded at pH 3, 5, 7 in the presence of 0.1, 1, 5, 10 mM CaCl_2 or KH_2PO_4 (data shown only at pH 5, 5 mM CaCl_2 or KH_2PO_4). At pH 7 a minor change in the intensity with λ shift was observed. However, in presence of Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$ no significant change was observed. This data was analyzed to provide estimation of secondary structural elements by different methods: CONTINN, SELCON3 and K2D (33,34). The method SELCON3 did not provide any results whereas K2D reports data with very high error. The CONTINN gave an estimation of α -helix (4.2%), β -sheet (30.2%), β -turn (10.7%), PP2 (12.3%), unordered (42.6%), RMSD (0.048), NRMSD (0.155).

Microscopic analysis of particles

The possibility of formation of more organized structure like nanospheres formed by amelogenin was also taken into account for these peptides. In the recorded AFM images, M28D showed presence of nanostructures of diameter 48.9 (± 8.3 , $n=10$) nm and height 2.0 (± 0.43 , $n=10$) nm at pH 3, and of diameter 54.4 (± 6.7 , $n=10$) nm and height 3.2 (± 0.64 , $n=10$) nm at pH 7 (Fig. 7A,B), the p -value was 0.055 for the t-test, indicating that the effect of pH on particle size is statistically insignificant. M17P also showed irregular shaped larger nanostructures of an average diameter 54.9 (± 10.5 , $n=10$) nm and height 4.8 (± 0.92 , $n=10$) nm at pH 3, and of diameter 63.7 (± 12.7 , $n=10$) nm and height 5.9 (± 1.9 , $n=10$) nm at pH 7, the p -value was 0.06 for the t-test, indicating that the effect of pH on particle size is statistically insignificant. No conspicuous structures were observed for these peptides at pH 5, and at any pH for S11D distinguished from control images (data not shown).

Discussion

The significance of amelogenin in the regulation of growth, shape and arrangements of enamel crystals is well documented (1). Amelogenin, which constitutes 90% of the developing matrix, readily processed to smaller fragments such as 20 kDa, 23 kDa and TRAP (tyrosine rich amelogenin peptides). Earlier, formation of nanospheres, involving hydrophobic core and excluding N- and C-termini, found to be crucial to the regulatory function of amelogenin. (36–38) However, the recent observation of self-aligning nanoribbons in an oil-water system also indicated possibilities of other supramolecular structures of amelogenin that may be able to guide apatite crystallization during enamel development (10). SS-NMR studies have indicated that the charged C-terminus is oriented towards the HAP surface (24). In porcine amelogenin, it was observed that the C-terminal 12 amino acids, defined as teleopeptide, strongly influenced apatite binding (20). On the other hand, the affinity to apatite was considerably lowered, when synthetic C-terminal decapeptide was examined in isolation. This finding concluded the role of overall amelogenin conformation in the control of apatite affinity conferred by C-terminus (23).

In the present investigation instead of a random selection of C-terminal teleopeptide, *in vivo* MMP-20 cleavage sites at the C-terminal were considered to design possible fragments as reported for recombinant pig amelogenin (5,18). The constructed peptides on the hydropathy scale appeared as bipolar (M28D), hydrophobic (M17P) and strongly hydrophilic (S11D) (Fig. 1B), which possibly resulted in differences in their properties.

In certain cases, ζ -potential was found to be associated with aggregation propensity of the peptides as observed for the recombinant amelogenins (29). Unlike the longer amelogenin proteins, where aggregation was found in the vicinity of their pIs (29;39), these peptides showed high propensity for aggregation at pH 7, about 2–3 pH units above their pIs, when they were negatively charged. Whereas, their positively charged state ($\text{pH} < 4$) was less prone to aggregation. Addition of Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$ ions caused a rapid drop in ζ -potential, usually associated with the aggregation. This effect on the ζ -potential and particle size occurred at CaCl_2 or KH_2PO_4 concentration less than 1 mM. Above this point, the effect seemed to be saturated, indicating that after specific adsorption of Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$ the peptides agglomerates reorganize in some way that causes either decrease in net positive or increase in net negative surface charge. A similar behavior has also been reported for full-length amelogenin (40) and BSA (data not shown), both of them also interact with Ca^{2+} (4).

Addition of Ca^{2+} or $\text{H}_x\text{PO}_4^{x-3}$ also resulted in quenching of the tryptophan fluorescence of M28D and M17P, which was used to calculate the affinity of these ions to the peptides. The binding affinity constants were not high (millimolar range), compared to other proteins and enzymes for their targets- substrates or inhibitors (micromolar to nanomolar range).

However, they were still comparable to rH174 ($K_a = 1332 \text{ M}^{-1}$) and rLRAP ($K_a = 8551 \text{ M}^{-1}$) (4). Interestingly, it was found that removal of the last 11 aa from rH174, resulting rH163, actually increases its affinity for mineral ions, whereas, further truncation of 17 aa, resulting in rH146, decreases its affinity. Similar results were obtained for LRAP, a product of alternatively splicing of amelogenin containing only first 32 and last 26 aa of rH174, thus missing the central 116 aa, which showed 6.4 times higher affinity than rH174 (4). One possible reason, for the difference in the affinity of rH174 and rH163, is that the presence of both, four negative and four positive charges, in this 11 amino acid region, may lock some water molecules which may prevent this residues to react with mineral ions (41). This 11 aa region, however also present in LRAP, does not affect its ion binding. This anomaly could be due to the reason that rH174 forms nanospheres with the C-terminals on the particle surface and in close proximity of each other, creating a strong ion shield that might be absent in LRAP. Further truncation of 17 residues results in amelogenin rH146, which shows very poor affinity that may be associated with the overall loss of all available ionic contacts in the last 28 residues region of rH174. It also substantiates the importance of C-terminus in the ion binding property of amelogenins. These observations indicate that ionic affinity of amelogenins, however conferred by C-terminus, depends on overall conformation of the protein molecules and is not just subjected to the numbers of charged residues.

The overall relatively low binding affinity of amelogenin and its derivatives is expected, and conforms to their speculated *in vivo* functionality. It seems, that during enamel formation amelogenin and/or its derivative guide the incoming ions by loosely holding them allowing a transfer of ions to the growing apatite crystal. Tight mineral binding would not be beneficial, since the presence of a protein with high affinity to calcium and phosphates will remove these ions from the bulk solution and may inhibit apatite formation as shown for some phosphorylated proteins (osteopontin and osteocalcin) (42,43). The thermodynamic properties of rH174 for Ca^{2+} indicates that the binding is carried out by weaker ionic interactions, and excludes presence of van der Waals forces (4). The C-terminal peptides contain several positive (Arg, Lys) and negatively (Asp, Glu) charged amino acids, which could be possible sites for the mineral ions bindings.

The peptide M28D and M17P were found to be unordered in secondary structure analysis using circular dichroism (CD), but also showed some unidentified negative peak at 235 nm. No change in their secondary structure was reported with the addition of calcium or phosphate, similarly as observed for LRAP (4) and in contrast to certain proteins, like calmodulin, which is unordered in solution but attains well-ordered conformation upon binding to Ca^{2+} (44). Interestingly, the shortest peptide S11D, which lacks 17 amino acids from its N-termini compared to M28D, showed striking difference in the secondary structure. The peptide has some amount of secondary structures mainly β -sheets and -turns. Certain peptides have been reported to form stable β -hairpins by intermolecular interaction, (45) which could further arrange as sheets, or may act as nucleation site for the aggregation to form larger particles.

The peptide M28D and M17P formed conspicuous nanostructures at pH 3 and 7 and but not at pH 5. Near their pI at pH 5 they tend to aggregate non-specifically and failed to form any noticeable structures. M28D formed more definite and uniform nanostructures, which could be attributed to its amphiphilic nature, where hydrophobic forces tend to clump together and the hydrophilic part stabilizes the newly formed structure from further aggregation or precipitation (46). The peptide M17P, relatively hydrophobic, showed irregular nanostructures. However, the size of the particle observed in DLS does not match the structures seen in AFM at pH 7 for both the peptides. Immobilization of the larger protein particles (700–1000 nm, as observed in DLS) on the glass surface, during the AFM disc, preparation may result in re-dispersion of smaller nanosized subunits, due to weak attraction

between the peptide nanostructures. The failure to observe nanostructures for S11D, in AFM, seems to be associated with its highly hydrophilic charged nature, where opposite charges may attract each other to form larger structure with definite secondary structures as seen in DLS and CD spectrum, respectively, but such structures held together by weaker forces may be re-dispersed during AFM sample preparation. A similar effect has been described for the full-length amelogenin which shows large particle sizes by DLS but mainly the presence of 20–30 nm sized spheres when observed by AFM (29).

In conclusion, it was observed that the C-terminal peptides (a) are able to bind calcium and phosphates; (b) can make nanostructures; and (c) can attain more ordered secondary structures. Hence, it may be speculated that they may act as carrier of these ions to possible nucleation and growth sites of apatite mineral.

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A

B

Fig. 1. Sequence of peptides. (A) Sequence of full length human recombinant amelogenin (rH174) showing the MMP-20 cleavage position (/), and the sequence of the peptides (---). Acidic residues in peptides are marked as red bold, and basic residues are underlined blue. (B) Hydropathy profile of the C-terminus prepared according to KYTE & DOOLITTLE (47) using window size 3.

Fig. 2. The average particle size and ζ -potential of aqueous solution of the peptides. The pH was achieved by adding 0.1M HCl/KOH. Error bars indicate SD from 3 sets of experiments.

Fig. 3. Hydrodynamic diameters and ζ -potentials of M28D (A & B), M17P (C & D) and S11D (E & F) in the presence of CaCl_2 (A, C & E) or KH_2PO_4 (B, D & F) in 10mM acetate buffer (pH 3–5) or 10mM Tris-HCl (pH 7) containing 50 mM KCl. Error bars represent SD from three sets of measurements (Panel A&B, empty circles, pH 3 particle size data, are masked by empty squares, pH 5 particle size data).

Fig. 4. Intrinsic fluorescence spectra of (A) M28D, (B) M17P, (C) rH174, (D) rH163, (E) rH146 excited at 280/295 nm in 10mM acetate buffer (pH 3–5) or 10mM Tris-HCl (pH7–8.4) containing 50 mM KCl.

Fig. 5.

Determination of affinity constants for protein-calcium interactions by fluorescent spectroscopy for (A) M28D, (B) M17P, (C) rH174 (D) rH163 and (E) rH146. The peptides (1 mg/ml) and proteins (0.25 mg/ml) in 200 μl of 10mM Tris-HCl at pH 7 (M28D, M17P)/ 7.4 (rH174)/ 8 (rH163)/ 8.4 (rH146) containing 50 mM KCl were excited at 280/295 nm in the absence and presence of (0–10 mM) CaCl_2 at 25 $^\circ\text{C}$. F_0 and F_∞ are the fluorescence intensity of free protein and bound to calcium at infinity calcium concentration, respectively; and F_C is the fluorescence intensity at given calcium concentration $[C]$. Inset represents progressive fluorescence quenching after the addition of CaCl_2 aliquots.

Fig. 6. The far-UV circular dichroism (CD) spectra of peptides. The peptides 0.5–1 mM (A) M28D, (B) M17P and (C) S11D in 10mM acetate buffer (pH 3–5) or 10mM Tris-HCl (pH 7) containing 50 mM KCl in the absence of $\text{Ca}^{2+}/\text{H}_x\text{PO}_4^{x-3}$ and in the presence of 5 mM $\text{Ca}^{2+}/\text{H}_x\text{PO}_4^{x-3}$ (data only at pH 5 shown).

Fig. 7. (A-C) AFM topographic image of (A) M28D at pH 3, (B) M28D at pH 7, (C) M17P at pH 3 and (D) M17P at pH 7. The peptides (1mg/ml) in 10mM acetate buffer (pH 3) or 10mM Tris-HCl (pH 7) containing 50 mM KCl were allowed to settle on AFM discs for 30 min, washed with water and dried by compressed air.

Table 1

Affinity constants for peptide-mineral interactions.

Ligand	M28D $K_a(M^{-1})$	M17P $K_a(M^{-1})$	rH174 $K_a(M^{-1})$	rH163	Rh146
Ca^{2+} at pH 3	218 (± 23)	701 (± 24)	822 (± 42)	ND	ND
Ca^{2+} at pH 5	544 (± 46)	958 (± 46)	1077 (± 90)	3876 (± 117)	159 (± 71)
Ca^{2+} at pH 7-8.4	1132 (± 40)	1428 (± 65)	1538 (± 137)	3993 (± 191)	625 (± 151)
$H_2PO_4^{x-3}$ at pH 3	188 (± 21)	ND	ND	774 (± 194)	ND
$H_2PO_4^{x-3}$ at pH 5	467 (± 69)	1068 (± 47)	1168 (± 123)	2394 (± 180)	160 (± 79)
$H_2PO_4^{x-3}$ at pH 7-8.4	1314 (± 141)	(596 ± 12)	750 (± 58)	1041 (± 153)	966 (± 134)

All data were collected in 10 mM acetate buffer (pH 3-5) or Tris-HCl buffer (pH 7 for M28D & M17P, pH 7.4 for rH174, pH 8 for rH163, pH 8.4 for Rh146) containing 50 mM KCl at 25 °C.

ND- no change in fluorescence detected.

Values in parentheses represent SD (n=3).