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DPPI MAY ACTIVATE KLK4 DURING ENAMEL FORMATION

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Abstract

Kallikrein-4 (KLK4) is a serine protease expressed during enamel maturation and proteolytic processing of the enamel matrix by KLK4 is critical for proper enamel formation. KLK4 is secreted as an inactive zymogen (pro-KLK4) and identification of its activator remains elusive. Dipeptidyl peptidase I (DPPI) is a cysteine aminopeptidase that can activate several serine proteases. In this study we sought to examine DPPI expression in mouse enamel organ and determine if DPPI could activate KLK4. Real-time PCR showed DPPI expression throughout amelogenesis with highest expression at maturation and immunohistochemical staining of mouse incisors confirmed DPPI expression by ameloblasts. We demonstrate *in vitro* that DPPI activates pro-KLK-4 to cleave a fluorogenic peptide containing a KLK-4 cleavage site. Examination of mature enamel from DPPI null mice by FTIR showed no significant accumulation of protein; however, microhardness testing revealed that loss of DPPI expression significantly reduced enamel hardness.

INTRODUCTION

Dental enamel is formed in three major stages. During the secretory stage, the ameloblast cells secrete large amounts of proteins (amelogenin, ameloblastin, enamelin) and matrix metalloproteinase-20 (MMP20) which cleaves these enamel proteins. The ameloblasts then go through transition and maturation phases where they secrete the serine protease kallikrein-4 (KLK4) (Hu et al., 2000; Hu et al., 2002). KLK4 further degrades the enamel proteins which are ultimately removed from the enamel matrix resulting in virtually protein-free mature enamel. Proteolytic processing of the enamel matrix by KLK4 is critical for proper enamel formation and homozygous mutation of KLK4 causes *Amelogenesis Imperfecta* characterized by enamel that is hypomineralized and protein-rich (Hart et al., 2004; Wright et al., 2006).

KLK4, like other kallikreins, is secreted as an inactive zymogen. These serine proteases are synthesized as prepro-enzymes that are proteolytically processed to secreted pro-forms via removal of their signal peptide. The inactive pro-KLKs are then activated extracellularly by specific release of their amino-terminal propeptide. Activation of kallikrein family pro-forms has been well studied (Yoon et al., 2007; Emami and Diamandis, 2008); however, identification of the KLK4 activator remains elusive. The tooth specific protease MMP20 has been shown to activate KLK4 *in vitro* (Ryu et al., 2002); however, KLK4 and MMP20 expression overlap only briefly during enamel development (Hu et al., 2002). KLK4 is secreted throughout maturation and is not autocatalytic which suggests another protease activates KLK4 *in vivo*.

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KLK4 is also expressed in prostate and ovaries and is overexpressed in prostate and ovarian cancer (Dong et al., 2001; Klokk et al., 2007). Normal MMP20 expression within these tissues has not been demonstrated indicating the strong possibility of additional activator(s) of KLK4.

Dipeptidyl peptidase I (DPPI), also known as cathepsin C, is a ubiquitously expressed cysteine aminopeptidase that sequentially removes two N-terminal amino acid residues from proteins. In addition to its lysosomal activity, DPPI can be secreted (Wolters et al., 1998) and activates proenzymes of chymotrypsin-like serine proteases. DPPI activates granzymes A and B *in vivo* (Pham and Ley, 1999) as well as cathepsin G, elastase (Adkison et al., 2002) and chymases (Wolters et al., 2001).

In this study we sought to characterize DPPI expression within mouse enamel organ and determine if DPPI could activate pro-KLK4. We also examined enamel from DPPI-null incisors to determine if DPPI is essential for normal enamel development.

MATERIALS AND METHODS

All animals used in this study were housed in an Association for Assessement and Accreditation of Laboratory Animal Care accredited facilities.

Real-time PCR Analysis of Gene Expression in Mouse Enamel Organ

Mouse molars were harvested at 3, 5, 7, 9 and 11 days post-natal and subjected to qPCR analysis by iQ SYBR green (Bio-Rad). RNA was extracted and reverse transcribed as previously described (Turk et al., 2006). Gene-specific primers were designed using DNAStar software. Primers for DPPI expression were: forward (5'-GGTTGTATCTTGCAGCCCCTATG-3'); reverse (5'-TTCTTCCACCACCCCAAAATCTT-3'). The PCR temperature profile was 3 min at 95°C, 20s at 95°C, 30s at 64°C for 45 cycles, and 30s at 95°C for 1 cycle followed by stepwise increases to generate the melt curve. Results were normalized to EF1α1 RNA expression as previously described (Pfaffl, 2001) and are presented as fold increase in expression over day 3 levels. Data is representative of 6 individual mice per time point with measurements for each mouse repeated twice. Statistical significance of each time point compared to day 3 (secretory stage) was analyzed by Bonferonni's Multiple Comparison Test.

Immunohistochemistry of Mouse Incisors

Immunohistochemical analysis of demineralized, paraffin-embedded and sectioned mouse mandibular incisor was performed. Sections were incubated in 10 mM sodium citrate buffer, pH 6.0 for 10 min at 92°C for antigen retrieval and in blocking agent for 20 min followed by overnight incubation in 10 μg/ml DPPI antibody (R&D Systems). Staining was visualized by incubation in peroxidase-conjugated antibody [Vectastain Elite ABC Kit (Goat IgG)] and Sigma Fast 3,3'-diaminobenzidine substrate. Sections were counterstained with 0.1% Fast Green and examined by light microscopy. A negative control section was not treated with the DPPI-specific antisera.

In vitro Activation of Recombinant KLK4

Inactive, recombinant human pro-KLK4 (0.1 μg/μl; R&D Systems) was incubated with active recombinant mouse DPPI (0.01 μg/μl; R&D Systems) in 50 mM MES, 50 mM NaCl, pH 5.5 at 37°C for 4 hours. As a positive control, the bacterial protease thermolysin was used to activate pro-KLK4. Inactive pro-KLK4 (0.1 μg/μl) was incubated with thermolysin (1 μg/ml; Sigma) in 50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, pH 7.5 for 2 hours at 37C.

To determine if KLK4 had become activated, activity was measured by incubation of 100 ng of DPPI- or thermolysin-activated KLK4 with 100 μM Boc-Val-Pro-Arg-AMC (R&D

Systems) in 100 μl of 50 mM Tris, pH 9.0 at room temperature in a 96 well-plate with agitation. Hydrolysis of the substrate was measured by monitoring fluorescence every 1 min for 2 hours with 380 nm excitation and 460 nm emission. Inactive pro-KLK4 (100 ng), active DPPI (100 ng) and thermolysin (10 ng) were also incubated with 100 μM fluorogenic peptide and fluorescence monitored.

DPPI Null Mice

DPPI$^{-/-}$ mice were generated in 129/SvJ, DBA/1J and C57BL/6 strains, as previously described (Pham and Ley, 1999; Hu and Pham, 2005; Pagano et al., 2007).

Fourier Transform Infrared Spectroscopy (FTIR)

Mandibular incisors were obtained from 3 DPPI null mice and 3 wild-type mice (C57BL/6 strain). The heads from euthanized mice were skinned and fixed in 4% neutral formalin/saline for 24h, then rinsed and incubated in phosphate-buffered saline at 4°C. Erupted portions of mandibular incisors from wild-type and DPPI null mice were embedded horizontally (to expose both enamel and dentin) in a hard-formulation epoxy embedding medium (EpoFix, EMS). Samples were polished to 0.25μm with a diamond suspension (EMS). Enamel and dentin from the erupted portions of incisors were studied in reflectance mode on a Multiscope FTIR microscope coupled with a Spectrum One FTIR spectrometer (Perkin Elmer). Spectra were collected from 4000 – 550 cm^{-1} at a resolution of 4 cm_{-1} with 64 accumulations per run. Background spectra were collected from a gold mirror. Reflectance spectra were processed using the Kramers-Konig transformation in Spectra 5.0 to obtain an absorbance-like spectrum. Corrected maximum heights of protein Amide I absorption peaks in the 1600 – 1700 cm^{-1} region and v_3 PO_4 absorption peaks in the 1000 – 1100 cm^{-1} region were measured. The Amide I/ v_3 PO_4 ratio was used to assess protein to mineral content in wild-type and DPPI null enamel. Statistical significance was determined by T-Test.

Vickers Microhardness Testing

The polished samples of 3 wild-type and 3 DPPI null incisors (described above) were tested for enamel microhardness on a Leco M 400 HI testing machine (Leco). Mandibular incisors from 2 additional strains of DPP$^{-/-}$ mice (129SvJ and DBA/1J) were also examined. Incisors from 2 mice of each strain were embedded and polished as described above.

Microhardness testing was performed with a load of 25 g for 5 sec with a Vickers tip. Thirty indentations per enamel sample were performed. Statistical significance was determined by T-Test.

RESULTS

DPPI is Expressed by the Enamel Organ and its Ameloblasts

Expression of DPPI with respect to enamel formation was examined by real-time PCR. DPPI expression was quantified at day 3, 5, 7, 9 and 11, where day 3 represents early secretory stage and day 11 represents maturation stage enamel organ. DPPI is expressed throughout amelogenesis and expression increased dramatically with an 11-fold increase at maturation over secretory stage (Figure 1).

Immunohistochemical analysis of mouse mandibular incisors with DPPI antibody was performed to confirm the ameloblasts express DPPI (Figure 2). Consistent with the qPCR results, DPPI is expressed within secretory and maturation stage ameloblast. DPPI expression is also detected within the odontoblast cells and pulp.

DPPI Activates KLK4 In Vitro

Activation of pro-KLK4 by DPPI was demonstrated by the use of a quenched fluorescent peptide. When the peptide is cleaved by active KLK4, it fluoresces and this fluorescence can be quantified. Specificity of KLK4 for the peptide was demonstrated by incubation of the quenched peptide with thermolysin and DPPI. Neither enzyme was capable of cleaving the peptide (Figure 3). Pro-KLK4 was also shown to be inactive and unable to cleave the peptide. After pro-KLK4 was incubated with DPPI, the peptide substrate was cleaved as indicated by the increase in fluorescence. Thermolysin, which was previously demonstrated to activate KLK4 *in vitro*, was used as a positive control.

DPPI Null Mice Have Decreased Enamel Hardness

FTIR analysis was performed to assess protein and mineral content. Mandibular incisors from wild-type and DPPI null C57BL/6 mice were examined by FTIR. The ratio between maximal intensities of protein Amide I and v_3PO_4 absorption bonds was used to assess the matrix to mineral ratio of the enamel. The DPPI null mice showed comparable results to wild-type with no significant difference in protein/mineral ratio (Figure 4A,B). The Amide I/v_3PO_4 maximum height ratio from the enamel of the DPPI null samples (0.0075 ± 0.0006) was not significantly different from the wild-type controls (0.0076 ± 0.0022). The dentin was also analyzed and no significant difference in the calculated Amide I/v_3PO_4 maximum height ratio was observed between wild-type (0.155 ± 0.006) and DPPI null mice (0.151 ± 0.003).

Microhardness of C57BL/6 DPPI null [324.2 VHN, SEM ± 16.70] enamel was significantly decreased ($p < 0.05$) as compared to control [403.7 VHN, SEM ± 29.86] (Figure 4C). The decrease in enamel hardness does not appear to be strain specific as 129SvJ [297.0 VHN, SEM ± 16.93] and DBA/1J [342.9 VHN, SEM ± 28.97] strain DPPI null mice also show decreased enamel hardness.

DISCUSSION

The activator of KLK4 *in vivo* remains uncertain. We therefore asked if DPPI, a widely expressed protease known to activate numerous serine proteases could also activate pro-KLK4. Herein we examine the expression of DPPI with respect to enamel formation and determine if DPPI null mice have defective dental enamel.

DPPI is expressed throughout enamel formation with expression increasing at transition stage and with even greater increases seen at maturation. This increase in expression coincides with the expression of KLK4, which begins to appear at transition and peaks at maturation.

Human prepro-KLK4 contains 254 amino acids, the inactive zymogen has 228 amino acids and the active protein has 224 amino acids. The amino acid sequence of the propeptide is not highly conserved across species; however, the propeptides of human, mouse, rat, pig, dog and cow contain amino acids that DPPI is able to cleave. DPPI cannot cleave dipeptides if there is a N-terminal Lys or Arg, or a P1 Pro residue (McDonald et al., 1969; McGuire et al., 1992). It is of note that active KLK4 from all these species begin with Ile-Ile. The active mature form of the chymases, cathepsin G and granzymes A and B, all of which are activated by DPPI, also begin with Ile-Ile. Using synthetic peptides, it was found that DPPI is unable to cleave Ile-Ile-AMC (Tran et al., 2002). This supports DPPI as a likely activator of KLK4 and our *in vitro* activity assay demonstrated that DPPI is capable of activating human pro-KLK4.

If DPPI is the sole activator of KLK4 *in vivo*, we would expect $DPPI^{-/-}$ mice to have inactive KLK4 and the phenotype to mimic KLK4 null mice. The enamel from KLK4 null mice have yet to be described; however, the enamel from individuals with *Amelogenesis Imperfecta* that have a mutation in the KLK4 gene do have a hypomaturation phenotype. Radiographically the

teeth appear morphologically normal in shape with only a slightly increase in X-ray opacity compared to dentin, indicative of a decreased enamel mineral content (Hart et al., 2004). Closer examination showed the enamel to be of normal thickness and prismatic (Wright et al., 2006).

We examined the mandibular incisors from DPPI null mice and while no decrease in mineral content was evident based on our FTIR analysis of protein to mineral ratio, the hardness studies showed that enamel quality was affected. The cleavage of enamel proteins by proteases is critical to crystal growth and any alteration to this cleavage will likely affect the overall structural properties of the enamel crystals. We propose that the loss of DPPI resulted in reduced activation of KLK4. While it appears the overall protein-to-mineral ratio is unaltered, the timing of protein removal, and therefore crystal formation is likely to have been affected. The result is the weakened or softer enamel seen by microhardness testing.

Loss-of-function mutations in the DPPI gene result in early onset periodontitis and palmoplantar keratosis, characteristic of Papillon-Lefevre syndrome (PLS) (Toomes et al., 1999; Hart et al., 1999; Pham et al., 2004). Very little information describing the enamel in PLS affected individuals can be found within the literature which may be attributed to the lack of dentition in many of these individuals. Typically, individuals with PLS shed all their deciduous teeth by the age of 4 and most secondary teeth are shed by age 14 (Hart and Shapira, 1994). Kerebel et al. performed an ultrastructural odontological study of a 12-year old female with PLS and noted structural abnormalities of the enamel including anomalies of the prism structure (Kerebel et al., 1975). A high degree of caries, which may be indicative of weakened or softened enamel, was also mentioned in the original identification of this syndrome by Papillon and Lefèvre (Papillon and Lefèvre, 1924). Radiographically, the teeth of PLS affected individuals appear normal. A more complete examination of enamel from PLS-affected individuals would be of great interest; however, the rarity of this syndrome (1-4 in 1,000,000) may make these studies difficult (Hart and Shapira, 1994).

Identification of the *in vivo* activator of KLK4 is important not only for furthering the understanding of enamel formation but also has exciting potential as a therapeutic target for both ovarian and prostate cancer. While it remains to be determined if DPPI is the predominant activator of KLK4 *in vivo*, this study provides the first evidence of a ubiquitously expressed protease that can activate KLK4.

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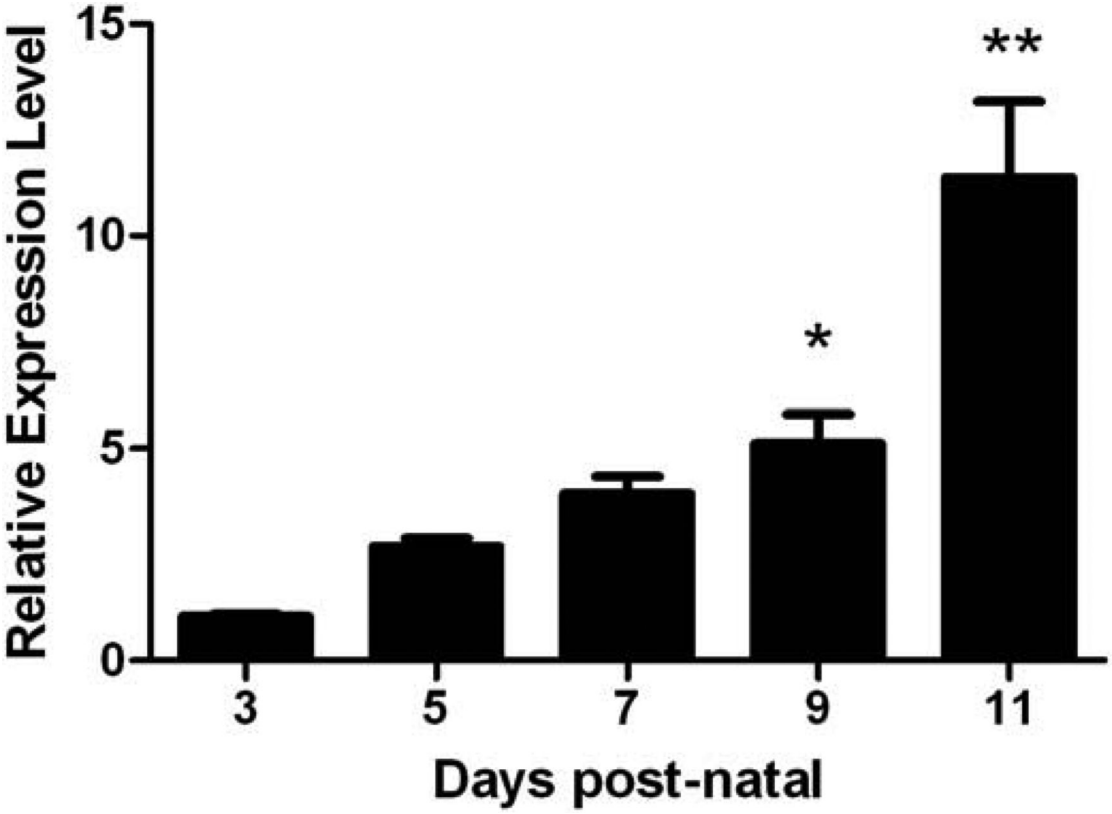

Figure 1. DPPI is Expressed in Enamel Organ

Mouse molars were harvested at defined time-points and subjected to real-time PCR analysis using DPPI specific primers. Expression was quantified at day 3, 5, 7, 9 and 11, where day 3 represents early secretory stage and day 11 represents maturation stage enamel organ. Data represents measurements of six individual mice per time point with each mouse repeated in triplicate (n=6). Results are presented as fold increase in expression over day 3 and normalized to EF1α1 mRNA expression. DPPI expression increases dramatically with an 11.38 ± 1.78-fold increase at maturation stage over secretory stage (* $p < 0.05$; ** $p<0.001$). Data are presented as mean ± SEM.

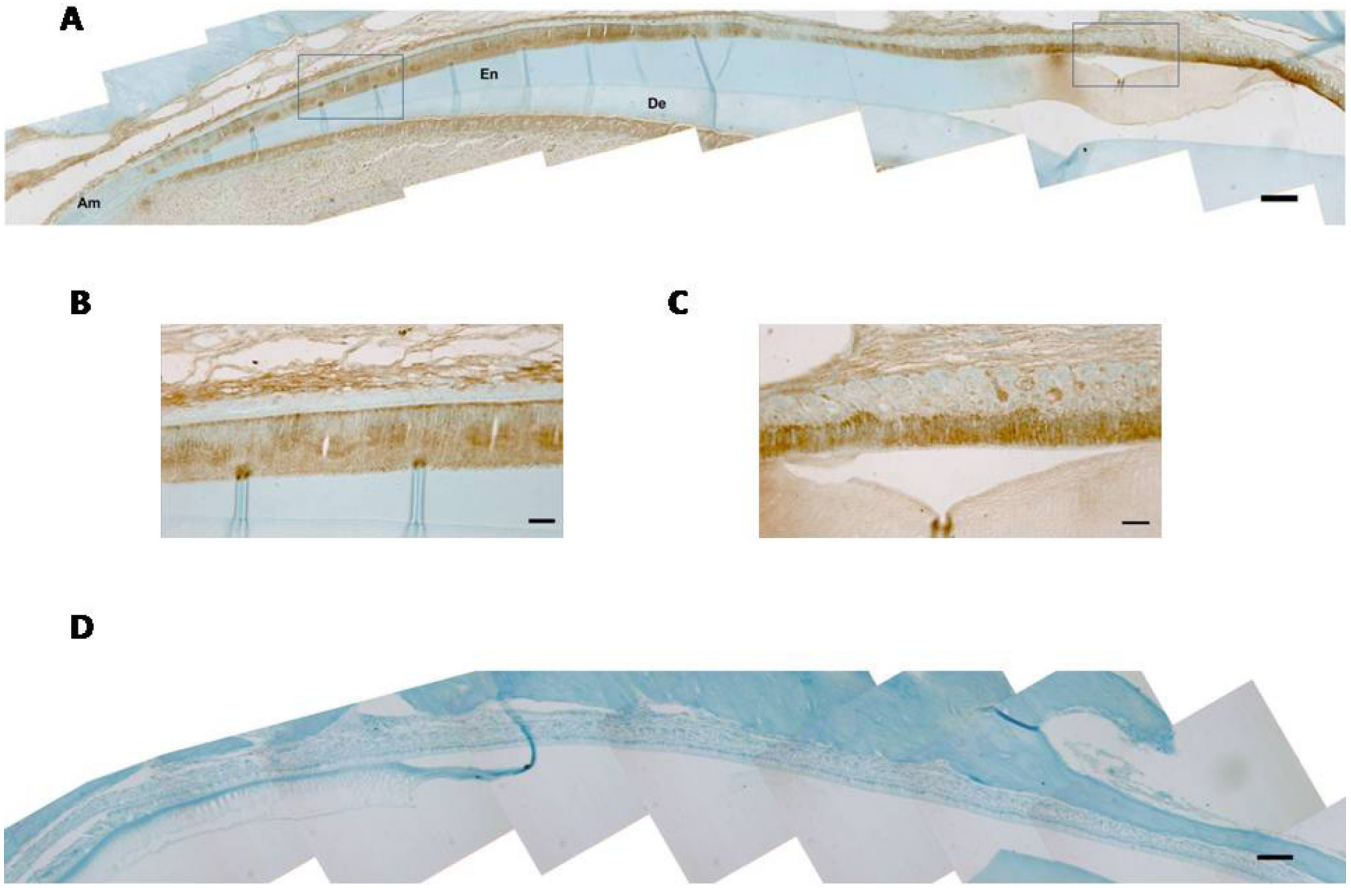

Figure 2. DPPI is Expressed by Ameloblasts

Immunohistochemical analysis of demineralized, paraffin sections of a mouse mandibular incisor counterstained with 0.1% Fast Green. Ameloblasts (Am), enamel space (En) and dentin (De) are depicted. A) Staining for DPPI in an adult mouse incisor (20X magnification). B) Enlargement of the indicated secretory stage ameloblasts from panel A (40X magnification). (C) Enlargement of the indicated maturation stage ameloblasts from panel A (40X magnification). (D) Negative control section treated with the secondary, but not primary antisera (20X magnification). Scale bar in panel A represents 140 μm for panel A. Scale bar in panel B and C represents 50 μm.

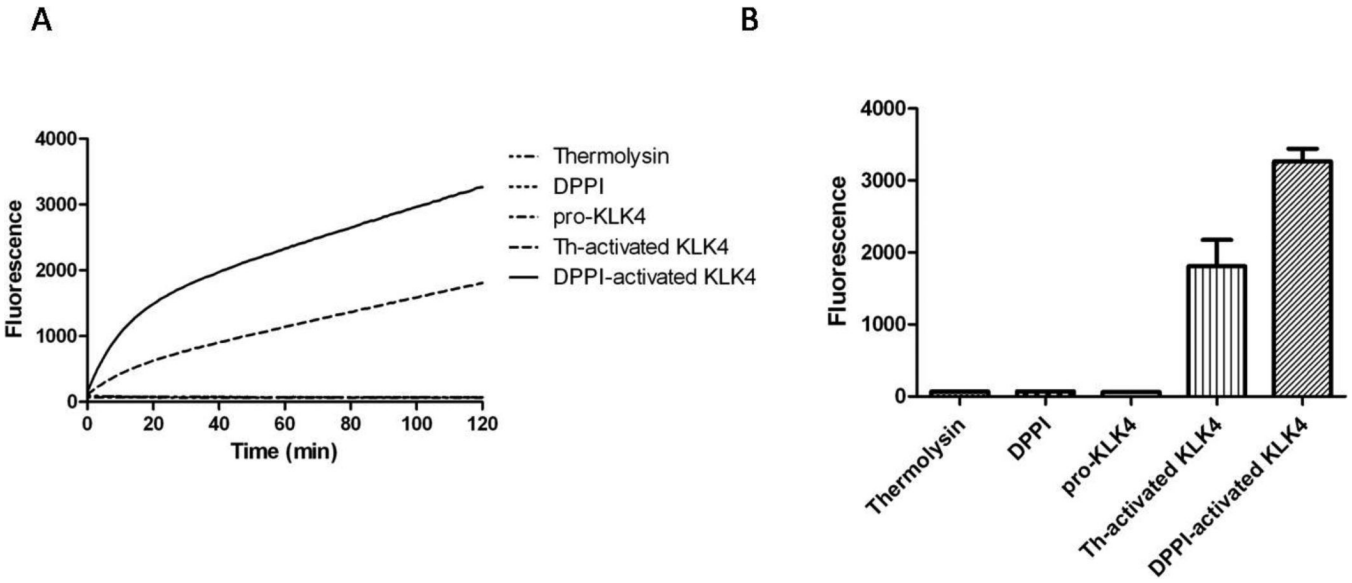

Figure 3. DPPI Activates Pro-KLK4 *In Vitro*

A) Hydrolysis of the KLK4 specific substrate (Boc-Val-Pro-Arg-AMC) was measured by monitoring fluorescence every 1 min over 2 hours with 380 nm excitation and 460 nm emission wavelengths. The fluorescent substrate was incubated with thermolysin, DPPI, pro-KLK4, thermolysin-activated KLK4 (th-activated KLK4) and DPPI-activated KLK4. B) Fluorescence measurement with the same enzymes after 2 hour incubation of the Boc-Val-Pro-Arg-AMC substrate (n=4). Incubation with thermolysin, DPPI or pro-KLK4 does not result in substrate cleavage. Thermolysin-activated KLK4 (th-activated KLK4) and DPPI-activated KLK4 both cleaved the substrate as indicated by the fluorescence. Data are presented as mean ± SEM.

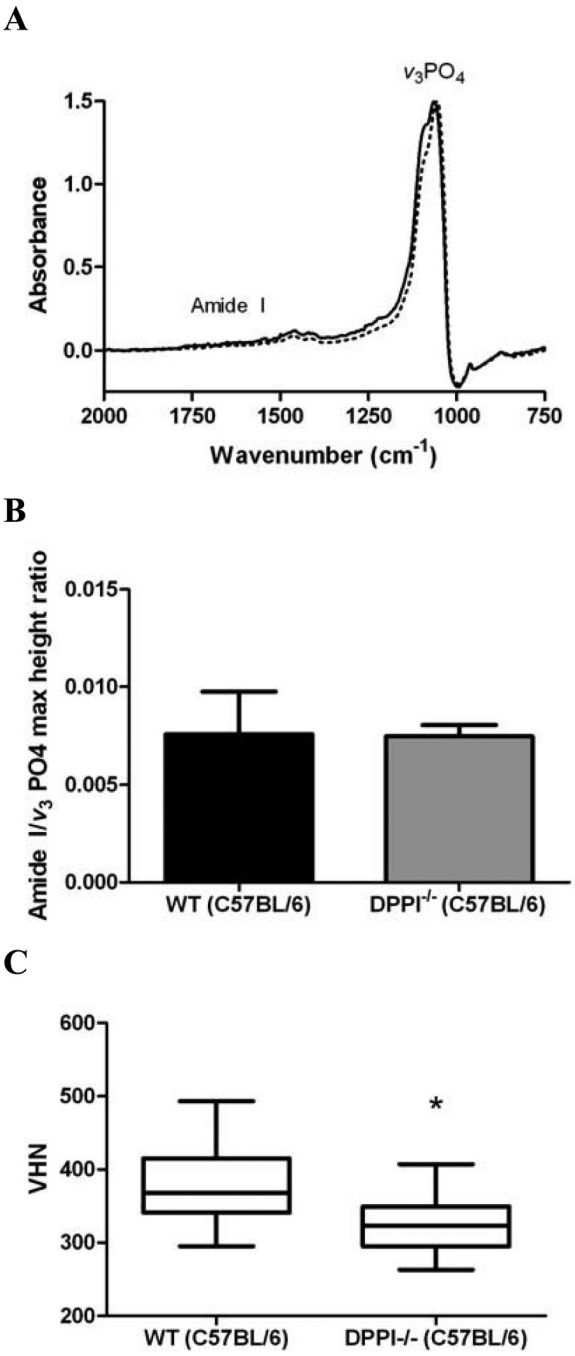

Figure 4. Characterization of Enamel from DPPI Null Mice

A) Representative normalized FTIR absorption spectra of enamel from wild-type (solid line) and DPPI null C57BL/6 mouse (dashed line) collected in reflectance mode. Amide I protein band and v_3 PO_4 band are shown. B) Amide I/ v_3PO_4 maximum height ratio from the enamel of DPPI null mice compared to wild-type C57BL/6 mice (n=3). No difference in protein to mineral ratio is observed in null mice. Data are presented as mean ± SEM. C) Box chart representing Vickers microhardness data for enamel from DPPI null and control incisors with 30 indentations per incisor (n=3). The Y-axis shows Vickers hardness number (VHN). Whiskers represent minimum and maximum. Student's t-test showed the means were significantly different (*, $p < 0.05$).