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Cleavage Site Specificity of MMP-20 for Secretory Stage Ameloblastin

APPENDIX

ISOLATION AND CHARACTERIZATION OF 23-kDa Ambn CLEAVAGE PRODUCT

Methods

Unerupted second molars were dissected from 6-month-old commercial pigs. The enamel organ epithelia (EOE) and dental pulp tissue were removed, and we obtained the outer enamel by shaving the enamel surface with a razor blade. The shavings were suspended in 50 mM Sörensen buffer (pH 7.4) with proteinase inhibitors (1 mM EDTA, 5 mM, 1,10 phenanthroline, and Protease Inhibitor Cocktail Set III; Novagen, Madison, WI, USA; 1 mM AEBSF, HCl, 0.80 μM Aprotinin, 50 μM Bestatin, 15 μM E-64, 20 μM leupeptin hemisulfate, and 10 μM Pepstatin A) and homogenized on ice. Insoluble material was pelleted by centrifugation, and the homogenization was repeated 2 more times. The supernatants were combined, and ammonium sulfate was added to 40% saturation. Insoluble material was pelleted by centrifugation. Ammonium sulfate was added to the supernatant to bring it to 65% saturation, and insoluble material was pelleted by centrifugation. The pellets were re-suspended in 0.5 M acetic acid. Insoluble material was removed by centrifugation. The supernatant was lyophilized, raised in 0.05% trifluoroacetic acid (TFA), and injected onto a TSK-gel ODS-120T column (4.6 x 25 cm, TOSH, Tokyo, Japan) for reversed-phase high-performance liquid chromatography (RP-HPLC) separation. Proteins were eluted with a linear gradient of 0-80% acetonitrile and 0.05% TFA. Individual peaks were collected, lyophilized, and characterized by SDS-PAGE and N-terminal sequencing (ABI 477A protein sequencer).

Results

The N-terminal sequence of the 23-kDa protein identified it as an ameloblastin cleavage product. The sequence (YGAMF PGFGGMRPNLGGMPPNSAKGGDFxLEFD) extended from Tyr^{223} to Asp^{255}, with Thr^{251} being a blank cycle.

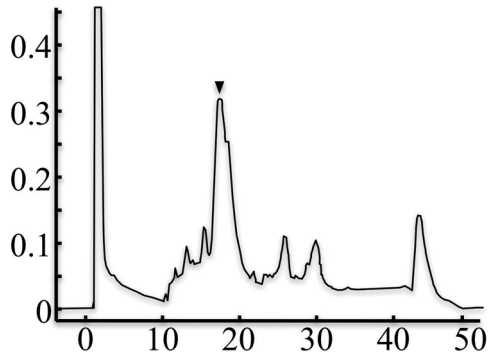
ISOLATION AND CHARACTERIZATION OF rAMBN, Klk4, AND MMP-20

Isolation and Characterization of Recombinant Ameloblastin (rAmbn)

We fabricated the expression construct by amplifying porcine ameloblastin cDNA from enamel epithelial tissue and ligating it to pEF6/V5-His-TOPO (Invitrogen) as described previously (Iwata *et al*., 2007). The rAmbn was secreted as a 440-amino-acid protein with a 45-amino-acid combined V5-epitope and poly-histidine tag fused to its C-terminus (KGNSADIQHSGGRS SLEGPRFEGKPIPNPLLGLDSTRTGHHHHHH).

HEK293-H cells (Invitrogen) were stably transfected with the pEF6/V5-His-TOPO Ambn construct with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen Gibco), with 10% (v/v) fetal bovine serum (FBS) (Invitrogen) in a 5% CO_2 humidified chamber. The medium was concentrated, and the highest-expressing HEK293-H line among 54 stable transfectants was identified by Western blot analyses (data not shown). The cells were seeded at a density of 7.8×10^4 cells/cm^2 and grown in CellStacks (Corning, Corning, NY, USA) as a monolayer. Once cells were confluent, DMEM was supplemented with 1% SerumPlus (SAFCO Bio-sciences, Lenexa, KS, USA) for daily collection of the medium. Cells were passaged weekly.

Recombinant ameloblastin was precipitated from the medium with 50% ammonium sulfate and proteinase inhibitors (EDTA 4 mM, pH 7.0, PMSF 0.5 mM) overnight on ice. After centrifugation, the precipitate was re-suspended in sodium phosphate buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.4) and applied to Talon Metal Affinity Resin (Clontech, Mountain View, CA, USA). The rAmbn was eluted with 200 mM imidazole, desalted by means of a centrifugal filter device (Amicon



Appendix Figure 1. The N-terminus of the 23-kDa ameloblastin cleavage product. **(A)** Chromatogram detected at 230 nm of proteins eluting from the RP-HPLC column. Arrowhead indicates peak and fraction containing the 23-kDa Ambn cleavage product. **(B)** SDS-PAGE stained with CBB showing the purified protein that was analyzed by N-terminal sequencing. M, molecular-weight marker; 1, contents of large peak eluting at 18 min; arrowhead points to 23-kDa Ambn product.

Ultra-15, Millipore, Billerica, MA, USA), and lyophilized. The protein was re-suspended in 0.05% trifluoroacetic acid (TFA) and injected onto RP-HPLC (Beckman Coulter, System Gold, Fullerton, CA, USA) in a C18 column, 25 cm × 10 mm × 5 µm (Discovery C18, Supelco, Bellefonte, PA, USA). As a starting buffer, 0.05% TFA was used, and 0.05% TFA + 80% acetonitrile was used as elution buffer with a linear gradient from 0-100%. The flow rate was 1.0 mL/min.

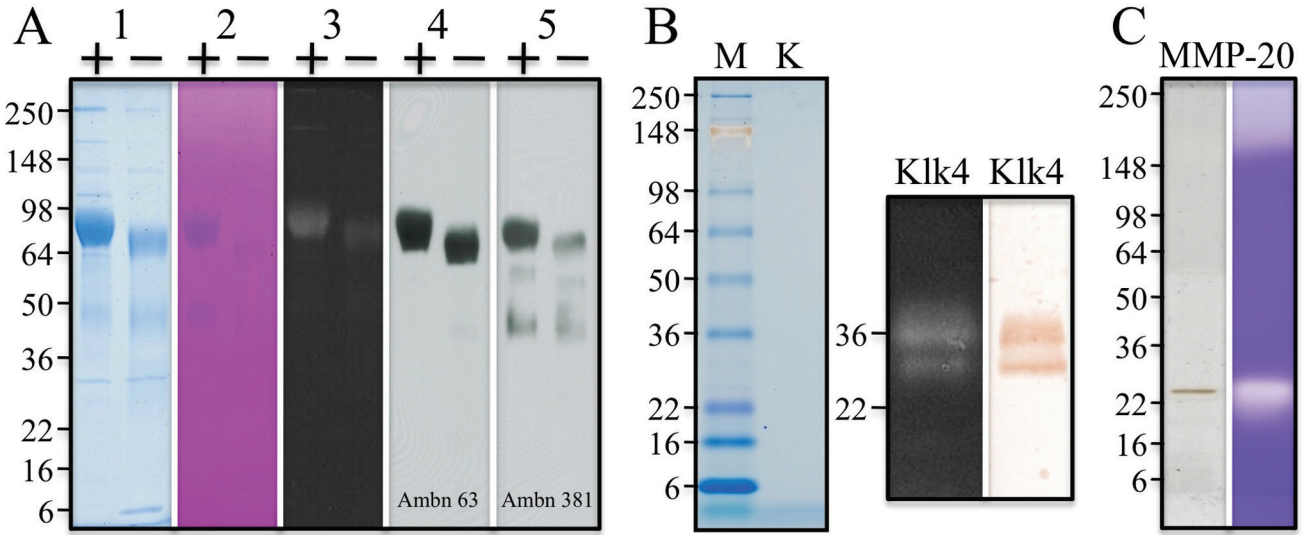
To demonstrate that the rAmbn was O-glycosylated, we incubated it with sialidase and O-glycosidase (QA-Bio, San Mateo, CA, USA) at 37ºC for 2 hrs. The sialidase was included because O-glycosidase cleaves only unsubstituted Gal-β(1-3) GalNAc-alpha chains attached to Ser or Thr. The digests were analyzed on SDS-PAGE stained with CBB, Stains-all, Pro-Q Emerald 488 (Molecular Probes, Eugene, OR, USA), and by Western blot analyses with N- and C-terminal specific Ambn antibodies (Fig. 2A, in appendix).

SDS-PAGE and Western Blotting

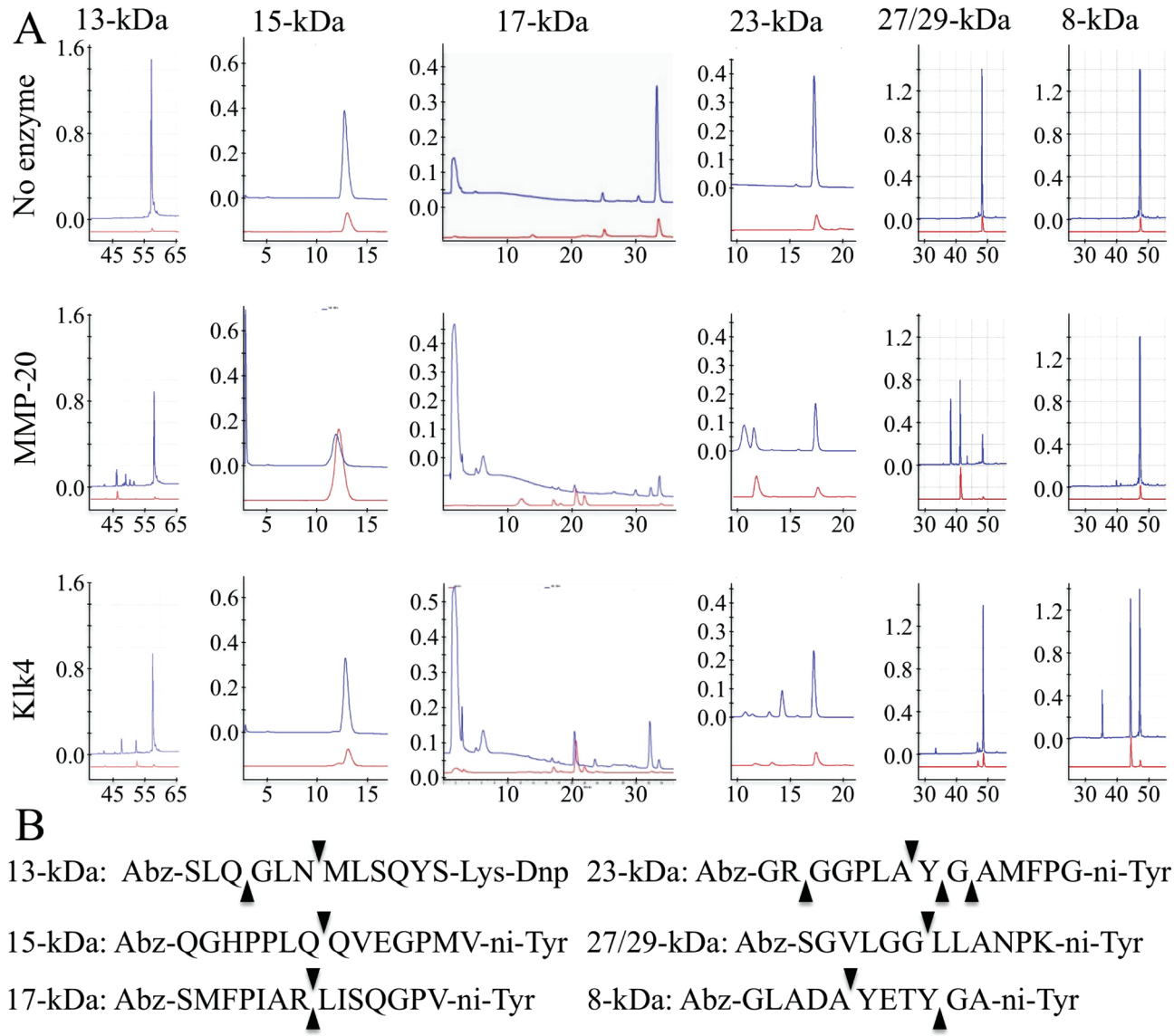
Samples were applied to pre-cast bis-(2-hydroxylethyl) amino-tris (hydroxy-methyl) methane (bis-tris) NuPAGE® gels (Invitrogen) run with MES buffer, then stained with Coomassie brilliant blue (CBB), Stains-all (Sigma, St. Louis, MO, USA), or Pro-Q® Emerald 300 Glycoprotein Gel Stain Kit (Invitrogen). After electrophoretic transfer of the proteins onto a nitrocellulose membrane (0.4 µm, Hybond™-ECL; GE Healthcare Biosciences, Little Chalfont, UK), blocking was performed with 5% non-fat dry milk for 1 hr, followed by incubation with a 1:1000 dilution of $Ambn^{63}$ antibody or a 1:5000 dilution of $Ambn^{381}$ antibody in Tween Tris-buffered saline (TTBS) containing 5% milk for 1 hr (Iwata *et al.*, 2007). The blots were washed 3x for 20 min each in TTBS and incubated with anti-rabbit IgG secondary antibody (BioRad, Hercules, CA, USA) at a dilution of 1:10,000. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL Plus; GE Healthcare Biosciences). Although the recombinant ameloblastin had been purified by affinity chromatography followed by RP-HPLC, there was still some contamination evident on SDS-PAGE (Fig. 2A), but the impurities were not characterized.

Isolation of Klk4 from Porcine Enamel

Klk4 was purified from unerupted 6-month-old pig second molars obtained from the Michigan State University Meat Laboratory (East Lansing, MI, USA) and processed as described previously (Yamakoshi *et al*., 2006). Hard-enamel shavings were



Appendix Figure 2. Isolated rAmbn, Klk4, and MMP-20. **(A)** (1) CBB-stained SDS-PAGE, (2) Stains-all-stained SDS-PAGE, (3) Pro Q Emerald 488-stained SDS-PAGE, and Western blots with the (4) $Ambn^{63}$ and (5) $Ambn^{381}$ antibodies. The + lanes are before deglycosylation with sialidase and O-glycosidase, and the - lanes are after deglycosylation. Note, in the deglycosylated lanes, that the recombinant Ambn shows a smaller apparent molecular weight, indicating that rAmbn is O-glycosylated. **(B)** CBB-stained SDS-PAGE showing that no protein is evident in a Klk4 sample, although the same amount of protease is readily detected on a gelatin zymogram and Western blot. **(C)** Silver-stained SDS-PAGE and casein zymogram showing the active catalytic domain of rMMP-20 used in this study.



Appendix Figure 3. Sites cleaved by MMP-20 and Klk4 in 6 fluorescent peptides. The FRET peptides containing a fluorescent signal (Abz-) on their N-termini and a quencher (-Lys-Dnp or nitro-Tyr) on their C-termini were digested by MMP-20 or Klk4. Half of the digestion was analyzed by RP-HPLC **(A),** and half was analyzed by mass spectrometry **(B).** (A) Chromatograms of FRET peptides before digestion (top row), after digestion with MMP-20 (middle row), and after digestion with Klk4 (bottom row). The peptides were detected by their absorbance of UV light (blue line). Fluorescence was detected as emitted light at 420 nm (red in chromatogram) after excitation at 320 nm. Following the digestions, the peak representing the intact peptide generally diminished, while smaller peaks were generated. Those with red peaks are N-terminal (Abz-containing) cleavage products. (B) Mass spectrometric analyses identified the cleavage products. Arrowheads above each peptide sequence indicate sites cleaved by MMP-20. Arrowheads below the peptide sequences indicate sites cleaved by Klk4. MMP-20 cleaved each peptide exactly at the sites corresponding to ameloblastin cleavages catalyzed *in vivo*: on the N-terminal sides of Met^{32}, Gln^{131}, Leu^{171}, Tyr^{223}, Leu^{301}, and Tyr^{343}.

homogenized in Sörensen buffer. Insoluble material was removed by centrifugation, and the supernatant was raised to 40% saturation with ammonium sulfate. The precipitate was removed by centrifugation, and the supernatant was raised to 65% saturation with ammonium sulfate. The precipitate containing Klk4 was pelleted by centrifugation and was re-suspended in 2 mL of resin buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4). Klk4 was bound to benzamidine sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) of the same volume on a rotor at 4°C overnight. After the beads were packed into a column, the bound protein was eluted with 5 mL of 0.05% TFA. The eluate was injected onto a C18 reverse-phase column (TSK-gel ODS-120T, 7.5 mm × 30 cm; TOSH) and eluted with a linear gradient (20-80% buffer B) at a flow rate of 1.0 mL *per* min. Buffer A was 0.05% TFA, and buffer B was 0.1% TFA in 80% aqueous acetonitrile. The Klk4 fraction was concentrated, and the buffer was exchanged with Tris (50 mM, pH 7.4) with a cellulose membrane (Ultracel, NMWL: 3000, Millipore); aliquots were stored at -80°C. No

protein bands were observed in a Klk4 aliquot on CBB-stained SDS-PAGE, but the Klk4 protease was detected as a doublet at 34 and 36 kDa by Western blotting and casein zymography (Fig. 2B).

Isolation of Recombinant MMP-20

Full-length porcine MMP-20 lacking only the signal peptide was expressed from the pProEx1 vector in bacteria (XL1-Blue; Stratagene, La Jolla, CA, USA), which fused the rMMP-20 to an N-terminal histidine tag as described previously (Bartlett *et al.*, 1998). When the optical density of the cells reached 0.6 at 600 nm, cells were induced to express protein by the addition of isopropyl-β-D-thiogalacto-pyranoside (IPTG; Roche, Indianapolis, IN, USA) to a final concentration of 0.4 mM. After 3 hrs of growth, the cells were pelleted by centrifugation, re-suspended in 4 M guanidine-HCl containing 50 mM Tris (pH 7.4), and protease inhibitor cocktail (1 tablet of Mini-complete EDTA-free, Roche, Mannheim, Germany), phenyl-methylsulfonyl fluoride (PMSF), 0.5 mM, DNase (200 μg/g bacteria), and sonicated on ice. The bacterial debris was pelleted by centrifugation, and the supernatant was filtered and incubated with Talon Metal Affinity Resin (Clontech, Mountain View, CA, USA) that had been equilibrated with sodium phosphate buffer containing 8 M urea. After 3 washes, rMMP-20 was eluted with 200 mM imidazole in sodium phosphate buffer containing 8 M urea and 0.5 mM beta-mercaptoethanol, pH 7.4. The eluant was desalted by means of a centrifugal filter device (Amicon Ultra-15, Millipore, Billerica, MA, USA) against 50 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 0.05 % Brij 35. The N-terminal histotag and propeptide and C-terminal hemopexin domain were removed by activation of the rMMP-20 with 10 mM calcium for 12 hrs at 37ºC. The non-catalytic cleavage products precipitated during the activation step and were removed by centrifugation. The purified 22-kDa rMMP-20 catalytic domain was characterized by SDS-PAGE and casein zymography (Fig. 2C).

Digestion of FRET Peptides

Six fluorescence resonance energy transfer (FRET) peptides were incubated with rMMP-20 or Klk4 (10-μL aliquot) for 36-48 hrs and analyzed by RP-HPLC in a C18 column (Fig. 3A), and the other half was analyzed by LC-MS/MS (NextGen Sciences, Ann Arbor, MI, USA) (Fig. 3B).

APPENDIX REFERENCES

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