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Identification of major matrix metalloproteinase-20 proteolytic processing products of murine amelogenin and tyrosine-rich amelogenin peptide using a nuclear magnetic resonance spectroscopy based method

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ABSTRACT

Objective: The aim of this study was to identify major matrix metalloproteinase-20 (MMP20) proteolytic processing products of amelogenin over time and determine if the tyrosine-rich amelogenin peptide (TRAP) was a substrate of MMP20.

Design: Recombinant ^{15}N-labeled murine amelogenin and ^{13}C,^{15}N-labeled TRAP were incubated with MMP20 under conditions where amelogenin self-assembles into nanospheres. Digestion products were fractionated by reverse-phase high-performance liquid chromatography at various time points. Product identification took advantage of the intrinsic disorder property of amelogenin that results in little change to its fingerprint ^{1}H-^{15}N heteronuclear single-quantum coherence nuclear magnetic resonance spectrum in 2% acetic acid upon removing parts of the protein, allowing cleavage site identification by observing which amide cross peaks disappear.

Results: The primary product in five out of the six major reverse-phase high-performance liquid chromatography bands generated after a 24 h incubation of murine amelogenin with MMP20 were: S55-L163, P2-L147, P2-E162, P2-A167, and P2-R176. After 72 h these products were replaced with five major reverse-phase high-performance liquid chromatography bands containing: L46-A170, P2-S152, P2-F151, P2-W45, and short N-terminal peptides. TRAP was completely digested by MMP20 into multiple small peptides with the initial primary site of cleavage between S16 and Y17.

Conclusions: Identification of the major MMP20 proteolytic products of amelogenin confirm a dynamic process, with sites towards the C-terminus more rapidly attacked than sites near the N-terminus. This observation is consistent with nanosphere models where the C-terminus is exposed and the N-terminus more protected. One previously reported end-product of the MMP20 proteolytic processing of amelogenin, TRAP, is shown to be an *in vitro* substrate for MMP20.

1. Introduction

Amelogenesis is a highly coordinated, dynamic, biomineralization process that is governed by the complex interplay between matrix proteins and solutes. The foremost enzyme to process the matrix proteins is matrix metalloproteinase-20 (MMP20) (Bartlett & Simmer, 1999; Ryu et al., 1999), a protease present at the initial stage of amelogenesis through to the early maturation-stage (Simmer & Hu, 2002). Mutations to MMP20 that inactivate the protein (Gasse et al., 2013; Kim et al., 2005) contribute to the autosomal recessive non-syndromic condition *amelogenesis imperfecta* (Wright et al., 2011), a disorder with clinical phenotypes that include hypomaturation, hypocalcification, and hypoplasia. The ablation of MMP20 has similar consequences in MMP$^{-/-}$ transgenic mice, thin and brittle enamel with an absent or malformed rod pattern (Caterina et al., 2002). The overexpression of MMP20 in MMP20$^{+/+}$ transgenic mice also leads to improperly formed enamel (Shin et al., 2014). Data suggests that this is due to excess β-catenin released by the MMP20 cleavage of cadherin cell-cell junctions in fibroblasts and epithelial cells near the ameloblasts (Shin, Suzuki, Guan, Smith, & Bartlett, 2016). The translocation of the β-catenin to ameloblast nuclei promotes premature cell migration that is responsible for the formation of soft enamel.

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One of the primary targets of MMP20 is amelogenin, the dominant protein in the first stage of amelogenesis. The full length protein is not the only amelogenin initially present as the RNA transcripts undergo extensive alternative splicing to generate amelogenin proteins of different lengths and combinations (Bartlett et al., 2006; Gibson et al., 1991). Just as it has been suggested that these amelogenin splice-variants may play a role in amelogenesis (Fincham, Moradian-Oldak, & Simmer, 1999), it has also been suggested that the cleavage products of amelogenin may perform essential functions in the process (Bartlett & Simmer, 1999; Yang, Sun, Ma, Fan, & Moradian-Oldak, 2011). Consequently, identifying the major MMP20 cleavage products of amelogenin may provide insights into understanding amelogenesis. Using a combination of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reverse-phase chromatography/mass spectrometric analyses, many of the MMP20 cleavage products of recombinant porcine and murine amelogenin have been identified *in vitro* and shown to mostly agree with the cleavage products observed *in vivo* (Ryu et al., 1999). However, mass spectrometry can be too sensitive, detecting the minor as well as the major products of proteolysis, with quantification difficult (Scalbert et al., 2009).

Here, a combination of high-performance liquid chromatography and nuclear magnetic resonance (NMR) spectroscopy was used to identify primarily the major products of MMP20 cleavage of amelogenin at a concentration of three mg/mL (0.13 mM) at pH 7.4, a condition shown to produce nanospheres (Bromley et al., 2011). Solution NMR spectrometry is especially useful for identifying amelogenin cleavage products because, as an intrinsically disordered protein (Delak et al., 2009), there is little change in the fingerprint ^{1}H-^{15}N heteronuclear single-quantum coherence spectrum of amelogenin in 2% acetic acid upon removing (Zhang, Ramirez, Liao, & Diekwisch, 2011), altering (Buchko & Shaw, 2015; Buchko, Lin, Tarasevich, & Shaw, 2013), or recombining (leucine-rich amelogenin protein, LRAP) (Buchko, Tarasevich, Roberts, Snead, & Shaw, 2010; Tarasevich et al., 2015) parts of the protein. Furthermore, the major product will be readily identifiable over any minor products due to concentration dependent intensity differences for each product in an ^{1}H-^{15}N heteronuclear single-quantum coherence spectrum. In addition to our studies with full length amelogenin, a method to prepare recombinant, ^{15}N- and ^{13}C-labelled tyrosine-rich amelogenin peptide (TRAP) is also described and used to unambiguously show that TRAP is a substrate for MMP20 *in vitro*. TRAP was first identified in ameloblasts in 1981 (Fincham, Belcourt, Termine, Butler, & Cothran, 1981) and is one of the major products of MMP20 digestion of amelogenin (Nagano et al., 2009; Ryu et al., 1999).

2. Materials and methods

2.1. Materials

Full length recombinant murine amelogenin (M179, P2-D180, the N-terminal methionine is removed in *Escherichia coli* by methionine aminopeptidase (Bonde & Bulow, 2012)) without an affinity tag was ^{15}N-labeled, expressed, and purified from *E. coli* following a previously described protocol (Buchko & Shaw, 2015). The catalytic domain of recombinant human MMP20 was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY) and 4–12% bis-tris polyacrylamide gels from Invitrogen (Carlsbad, CA). All chemicals were purchased from Research Products International Corporation (Mount Prospect, IL) except trifluroacetic acid (TFA), glacial acetic acid, and HPLC grade acetonitrile (Sigma Chemical Company, St. Louis, MI).

2.2. Cloning, expression, and purification of murine TRAP

The DNA encoding the primary amino acid sequence of murine TRAP (M1-W45) with a 21-residue, N-terminal extension (MAHHHHHHMGTLEAQTQGPGS-) for metal-affinity purification (nTRAP), was codon-optimized for *E. coli*, chemically synthesized, and inserted into the expression vector pJexpress414 (DNA2.0, Menlo Park, CA). The recombinant plasmid was then transformed into competent *E. coli* BL21(DE3) cells (Novagen, Madison, WI) following a heat shock method. To obtain uniformly ^{15}N-, ^{13}C-labeled nTRAP, the transformed cells (37 °C) were grown in 750 mL of minimal medium (Miller) containing $^{15}NH_4Cl$ (1 mg/mL), D-[$^{13}C_6$]glucose (2.0 mg/mL), NaCl (50 μg/mL), $MgSO_4$ (120 μg/mL), $CaCl_2$ (11 μg/mL), Fe_2Cl_3 (10 ng/mL) and the antibiotic ampicillin (200 μg/mL). When the cell culture attained an OD_{600} reading of ~0.8, it was transferred to a 25 °C incubator and protein expression induced with isopropyl β-D-1-thiogalactopyranoside (0.026 μg/mL). Approximately four hours later the cells were harvested by mild centrifugation and frozen at −80 °C.

To purify nTRAP, the frozen pellet was thawed, resuspended in ~25 mL of 8 M guanidinium hydrochloride, 100 mM $NaPO_4$, pH 8.2, and passed three times through a French press (SLM Instruments, Rochester, NY). Following 60 s of sonication the insoluble cell debris was pelleted by centrifugation (10 °C) at 25,000*g* for 60 min in a JA-20 rotor (Beckman Instruments, Fullerton, CA). The supernatant was then applied onto a ~25 mL Ni-NTA affinity column (Qiagen, Valencia, CA) pre-equilibrated with solubilization buffer (8 M guanidinium hydrochloride, 100 mM $NaPO_4$, pH 8.2). The column was washed stepwise by gravity (room temperature) with 40, 40, 40, 20, and 50 mL of buffer (6 M guanidinium hydrochloride, 100 mM $NaPO_4$, pH 8.2) containing 0, 10, 20, 50, and 500 mM imidazole, respectively. Recombinant TRAP eluted exclusively in the 500 mM imidazole fraction. This fraction was further purified by making it acidic with 50 μL of TFA (0.1%) and applied onto an XBridge Preparative C18 (5 μ, 10 × 250 mm) reverse-phase column (Waters, Milford, MA) pre-equilibrated with 100% Buffer A (0.05% TFA in water). The following gradient scheme was applied with Buffer B (70% aqueous CH_3CN in 0.05% TFA) and a flow rate of 2.5 mL/min: Step 1 – 0.5 Column Volume (CV), 100% Buffer A; Step 2 – 0.2 CV, linear gradient 0–45% Buffer B; Step 3 – 4.0 CV, linear gradient 45–85% Buffer B). The fractions containing nTRAP (~68% Buffer B) were pooled, frozen (−80 °C), and lyophilized. To effect removal of the N-terminal tag, lyophilized nTRAP was resuspended in 3C protease cleavage buffer (150 mM NaCl, 20 mM Tris, pH 7.6) at a concentration of ~1 mg/mL (yields were ~15 mg/L minimal media) and 3C protease added (1 μg per 50 μg target protein). This solution was cloudy and digestion under the standard protocol was extremely slow (standing overnight at 4 °C) (Choi et al., 2011). To accelerate cleavage, the reaction was performed at room temperature (~22 °C) with gentle agitation over 48 h with a second addition of 3C protease (1 μg per 50 μg target protein) after the first 24 h. The reaction mixture was then centrifuged, supernatant removed (little product present), and the pellet re-dissolved in 0.5–1 mL of 2% acetic acid prior to HPLC purification using the protocol described below for the MMP20 digestion products of full length amelogenin. The final lyophilized product was weighed and a 0.25 mM solution prepared in NMR buffer (2% CD_3CO_2D, 7% D_2O/91% H_2O, pH 2.8) for NMR characterization (Buchko, Bekhazi et al., 2008). Note that after 3C protease treatment the final product, rTRAP, contained four non-native residues (GPGS-) at the N-terminal and are labeled G1* through S4* in our discussions.

2.3. MMP20 digestions

For M179, a stock solution of ^{15}N-labelled protein (~20 mg/mL) was prepared by gently shaking purified protein in water for two days. The solution was filtered through a 0.2 μm microspin filter unit (Lida, Kenosha, WI) and the concentration measured by ultra-violet absorption spectroscopy using a 280 nm extinction coefficient of 20,300 $M^{-1}cm^{-1}$ (calculated). From this M179 stock solution a 3 mg/mL (0.13 mM) solution of M179 was prepared in 1 mL of MMP20 reaction buffer (25 mM Tris, 5 mM $CaCl_2$, pH 7.4). Following confirmation of the pH with a meter reading, 3 μg of MMP20 was added and the reaction gently agitated at room temperature using a Fisher Scientific Multipurpose tube rotator (10 rotations per minute). To monitor the reaction,