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ABSTRACT

Ameloblastin is processed by protease(s) during enamel formation. We tested the hypothesis that MMP-20 (enamelysin) catalyzes the cleavages that generate secretory-stage ameloblastin cleavage products. We isolated a 23-kDa ameloblastin cleavage product from developing enamel and determined its N-terminus sequence. Ameloblastin was stably expressed and secreted from HEK293-H cells, purified, and digested with MMP-20 or Klk4 (kallikrein 4). The digests were analyzed by SDS-PAGE and Western blotting, and cleavage products were characterized by N-terminal sequencing. Six fluorescent peptides were digested with MMP-20 and Klk4 and analyzed by RP-HPLC and by mass spectrometry. MMP-20 cleaved each peptide exactly at the sites corresponding to ameloblastin cleavages catalyzed *in vivo*. Klk4 cleaved ameloblastin and the fluorescent peptides at sites not observed *in vivo*, and cleaved at only a single correct site: before Leu¹⁷¹. We conclude that MMP-20 is the enzyme that processes ameloblastin during the secretory stage of amelogenesis, and we present a hypothesis about the sequence of ameloblastin cleavages.

KEY WORDS: proteases, enamel, tooth, fluorescent peptides.

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Cleavage Site Specificity of MMP-20 for Secretory-stage Ameloblastin

INTRODUCTION

Dental enamel is the product of ameloblasts and requires the secretion of 3 matrix proteins—amelogenin, ameloblastin, and enamelin—which are hydrolyzed by matrix metalloproteinase 20 (enamelysin, MMP-20) (Bartlett *et al.*, 1996) and kallikrein 4 (Klk4) (Simmer *et al.*, 1998) and removed from the matrix (Smith, 1998). Loss-of-function mouse models have been generated for each matrix constituent, and all exhibit severe enamel defects in the homozygous defective condition (Gibson *et al.*, 2001; Caterina *et al.*, 2002; Fukumoto *et al.*, 2004; Hu *et al.*, 2008; Simmer *et al.*, 2009). These components are individually necessary for proper dental enamel formation, but how they work together to establish the highly organized and mineralized enamel layer is unknown. It is also not known if the intact proteins and/or their cleavage products are important functionally.

Enamel formation progresses through a secretory and a maturation stage. MMP-20 is expressed during the secretory and early-maturation stages, while Klk4 expression starts in the transition between the secretory and maturation stages and continues throughout enamel maturation (Hu *et al.*, 2000; Simmer *et al.*, 2004), although PCR analyses have detected low levels of Klk4 mRNA in enamel organ epithelia dissected from the secretory stage (Nagano *et al.*, 2003). The cleavage of amelogenin during the secretory stage has been well-characterized for the pig (Yamakoshi *et al.*, 1994). Analyses of how MMP-20 and Klk4 cleave amelogenin *in vitro* indicate that MMP-20 can catalyze all of the main cleavages (Ryu *et al.*, 1999; Nagano *et al.*, 2009), while Klk4 catalyzes other cleavages that are not observed among the accumulated amelogenins in secretory-stage enamel (Ryu *et al.*, 2002).

Intact ameloblastin is a trace component of developing enamel and has never been isolated *in vivo*. Porcine ameloblastin is modified as it passes through the secretory pathway. The 395-amino-acid secreted protein (Ambn³⁹⁵) is O-glycosylated at Ser⁸⁶ (Kobayashi *et al.*, 2007) and Thr³⁴⁸ (Yamakoshi *et al.*, 2001), hydroxylated at Pro¹¹ (Hu *et al.*, 1997) and Pro³²⁴ (Yamakoshi *et al.*, 2001), and has 4 serines (Ser¹⁵, Ser¹⁷, Ser²⁰⁹, Ser²¹⁰) in the required context for phosphorylation by Golgi casein kinase (Brunati *et al.*, 2000). Because of these modifications, recombinant ameloblastin that is similar to the native protein must be expressed and secreted in eukaryotic cells (Zeichner-David *et al.*, 2006; Iwata *et al.*, 2007).

The *in vivo* pattern of porcine ameloblastin cleavages is not as well-defined as that of amelogenin, but a fair number of cleavage products and cleavage sites have been characterized. The initial cleavages of ameloblastin release 3 products from the ameloblastin N-terminal region. The 17-kDa product extends from Val¹ to Arg¹⁷⁰, the 15-kDa from Val¹ to Gln¹³⁰, and the 13-kDa from Met³² to Gln¹³⁰ (Fukae *et al.*, 2006). Cleavage after Leu³⁰¹ in the C-terminal region generates the 27- and 29-kDa calcium-binding proteins (Fukae and Tanabe, 1987; Murakami *et al.*, 1997) that are subsequently processed after Arg³¹⁹, Lys³⁷⁹ (Yamakoshi *et al.*, 2001), and Tyr³⁴³ (Yamakoshi *et al.*, 2006a), although the final cleavages may occur in later stages. Given the availability of active forms of MMP-20 and Klk4, and knowledge of the sites where ameloblastin is cleaved *in vivo*, we tested the hypothesis that MMP-20, but not Klk4, can catalyze the cleavages that generate ameloblastin products that accumulate during the secretory stage of amelogenesis, and present a hypothesis about ameloblastin cleavage progression as it may occur *in vivo*.

MATERIALS & METHODS

Protocol Approval

Animal protocols were reviewed and approved by the University of Michigan Institutional Animal Care and Use Committee.

Purification of the 23-kDa Ameloblastin

The 23-kDa ameloblastin cleavage product was isolated from the outer enamel shavings from the unerupted second molars of 6-month-old pigs. The protein was isolated from the neutral extract in the 40 to 65% saturation ammonium sulfate precipitate. The acid-soluble part of the pellet was fractionated by reversed-phase high-performance liquid chromatography (Appendix Fig. 1).

Expression, Purification, and Characterization of Recombinant Ameloblastin (rAmbn)

Recombinant porcine Ambn³⁹⁵ that had previously been transiently expressed as a secreted glycoprotein fused to a C-terminal V5-poly-histidine tag (Iwata *et al.*, 2007) was transfected into HEK293-H cells with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) and selected for stable integration with 0.3 µg/mL blasticidin S HCl (Invitrogen). The highest-expressing HEK293-H line among 54 stable transfectants was identified by Western blot analyses. Secreted rAmbn was isolated from the medium by precipitation with 50% ammonium sulfate, then re-suspended in sodium phosphate buffer and bound to a metal affinity column, eluted with imidazole, desalted, lyophilized, and finally purified by RP-HPLC. To demonstrate that the rpAmbn was O-glycosylated, we incubated it with sialidase and O-glycosidase (QA-Bio, San Mateo, CA, USA) at 37°C for 2 hrs. The sialidase was included because O-glycosidase cleaves only unsubstituted Gal-β(1-3)GalNAc-α 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 ameloblastin antibodies (Appendix Fig. 2A).

Isolation of Klk4 from Mature 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.*, 2006b). Klk4 was isolated from the neutral extract in the 40 to 65% saturation ammonium sulfate precipitate. The serine protease was isolated with benzamidine sepharose 4 Fast Flow beads, followed by RP-HPLC. 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 gelatin zymography (Appendix Fig. 2B).

Isolation of Recombinant MMP-20 (rMMP-20)

Porcine MMP-20 was expressed from the pProEx1 in bacteria fused to an N-terminal histidine tag (Bartlett *et al.*, 1998). The bacterial lysate was applied to a metal affinity column, washed, eluted, desalted/buffer-exchanged, and activated with 10 mM calcium. The purified rMMP-20 consisted of an active catalytic domain migrating at 22 kDa on a casein zymogram (Appendix Fig. 2C).

Digestion of rAmbn with rMMP-20 and Klk4

Full-length rAmbn was incubated with either rMMP-20 or Klk4 at an approximate enzyme-to-substrate ratio of 1:100 for various time intervals in 10 mM Tris-HCl with 10 mM Ca²⁺, pH 7.4, at 37°C. Reactions were quenched by the addition of sample buffer (Laemmli Sample Buffer, Bio-Rad, Hercules, CA, USA) and immediate freezing at -80°C.

N-terminal Sequencing

Digests of rAmbn were separated by SDS-PAGE and transblotted to a polyvinylidene fluoride (PVDF) membrane. The membrane was lightly stained with CBB, and selected bands were excised and sent to the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University for N-terminal sequencing.

Synthetic Ameloblastin Peptides

Fluorescence resonance energy transfer (FRET) peptides containing 11 to 14 amino acids of porcine ameloblastin sequences centering on known ameloblastin cleavage sites were commercially synthesized and purified (JPT Peptide Technologies, Berlin, Germany; Gen Script, Piscataway, NJ, USA). Separation of the fluorescence emitter (Abz) from the quencher (nitroTyr or Lys-Dnp) by enzymatic cleavages induced fluorescence (excitation at 320 nm and emission at 420 nm). Each peptide (100 µg) was suspended in 50 mM Tris, pH 7.4, 10 mM CaCl₂, and incubated with rMMP-20 or Klk4 (10-µL aliquot) for 36-48 hrs at 37°C in a

total reaction volume of 100 μ L. The reactions were stopped by the addition of sample buffer (Laemmli Sample Buffer, Bio-Rad) and freezing at -80°C . Half of each digest was analyzed by RP-HPLC in a C18 column, and the other half was analyzed by LC-MS/MS (NextGen Sciences, Ann Arbor, MI, USA).

RESULTS

A 23-kDa ameloblastin cleavage product was isolated from the outer layer of porcine secretory-stage enamel and shown to have the N-terminal sequence of YGAMFPGFGGMRPNLGGMP PNSAKGGDFxLEFD (Appendix Fig. 1), which demonstrates that ameloblastin is cleaved on the N-terminal side of Tyr²²³ *in vivo*. An expression vector engineered to express recombinant porcine ameloblastin was stably integrated into the HEK293-H cell line and used to isolate rAmbn. The recombinant protein stained positive with Emerald 488 and migrated faster on SDS-PAGE following digestion with O-glycosidase (Appendix Fig. 2A). This proves that the recombinant protein is O-glycosylated. Western blot analyses with anti-peptide antibodies against the segments near the N- and C- termini of ameloblastin were positive, indicating that the recombinant protein is full-length and not degraded (Appendix Fig. 2A).

The recombinant Ambn (rAmbn) was incubated with MMP-20 and Klk4 *in vitro*, and the digestions were analyzed by SDS-PAGE and Western blotting with Ambn⁶³ and Ambn³⁸¹ antibodies (Fig. 1). An important finding was that rMMP-20 digested rAmbn at multiple sites, but the early cleavages were always on the N-terminal side of the protein (Fig. 1). This can be inferred from the observation that only smaller bands (7- to 25-kDa) were detected by the Ambn⁶³ antibody, while larger products (mainly 45- to 65-kDa) were detected by the Ambn³⁸¹ antibody. Furthermore, the larger C-terminal products were Stains-all-positive, suggesting that they are calcium-binding.

The N-terminal sequences of 9 cleavage products from the rMMP-20 digestion and 5 from the Klk4 digestion of rAmbn were determined by N-terminal sequencing (Fig. 2). Five

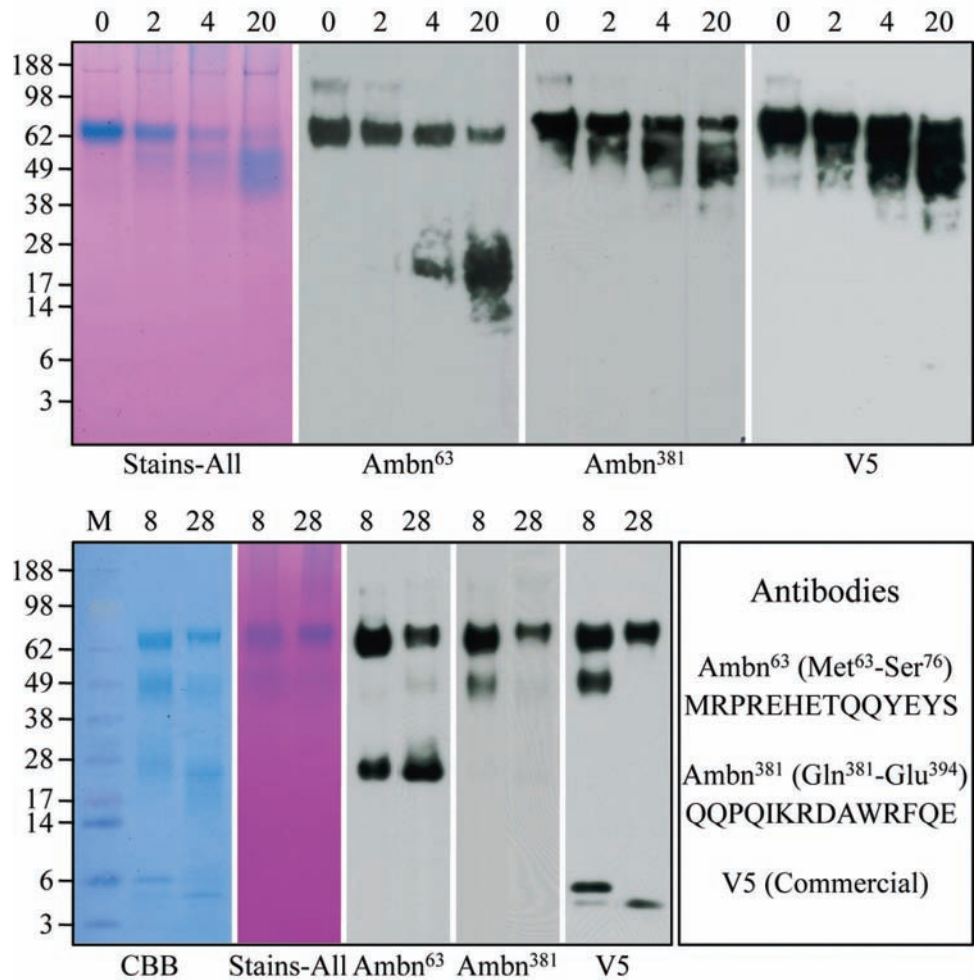


Figure 1. Digestion of rAmbn with rMMP-20 and Klk4. **(Top)** The rAmbn was digested by MMP-20 for 0, 2, 4, and 20 hrs. From left to right: Samples from these digests were separated by SDS-PAGE and stained with Stains-all, or transferred to a membrane for Western blotting with the primary antibodies Ambn⁶³, Ambn³⁸¹, or the V5 antibody, which recognizes the extreme C-terminus of the ameloblastin fusion protein. Note that the Ambn⁶³ antibody immunostained the intact protein at ~ 65-kDa and a smear of bands ranging from ~13- to 28-kDa, while the Ambn³⁸¹ and V5 antibodies recognized the intact protein and a smear of bands ranging from ~45- to 60-kDa. This shows that MMP-20 cleaved rAmbn at multiple sites on the N-terminal side of the protein. **(Bottom)** The rAmbn was digested by Klk4 for 8 and 28 hrs. From left to right: Samples from these digests were separated by SDS-PAGE and stained with CBB or Stains-all, or transferred to a membrane for Western blotting with the primary antibodies Ambn⁶³, Ambn³⁸¹, or the V5 antibody. The peptides used to synthesize the Ambn⁶³ and Ambn³⁸¹ are shown at the right.

MMP-20 products, ranging in apparent molecular weight from 22- to 37-kDa, gave the ameloblastin N-terminal sequence, 1-VPAFPRQP. The larger MMP-20 cleavage products (48- to 57-kDa) were cleaved on the N-terminal sides of Tyr²²³, Leu¹⁷¹, and Gln¹³¹, respectively. All of these cleavages are known to occur *in vivo*. Analysis of these data, taken together, suggests that rMMP-20 cleaves rAmbn initially at 3 sites, releasing the following pairs of cleavage products: N-terminus to Ala²²²/Tyr²²³ to C-terminus; N-terminus-Arg¹⁷⁰/Leu¹⁷¹ to C-terminus; and N-terminus to Gln¹³⁰/Gln¹³¹ to C-terminus. Klk4, in contrast, cleaves rAmbn on the N-terminal sides of Gly²⁹, Ser⁴⁴, Leu¹⁷¹, Met¹⁹², and Phe³⁹². Only the cleavage at Leu¹⁷¹ has been observed *in vivo*, and MMP-20 also cleaves at this site.

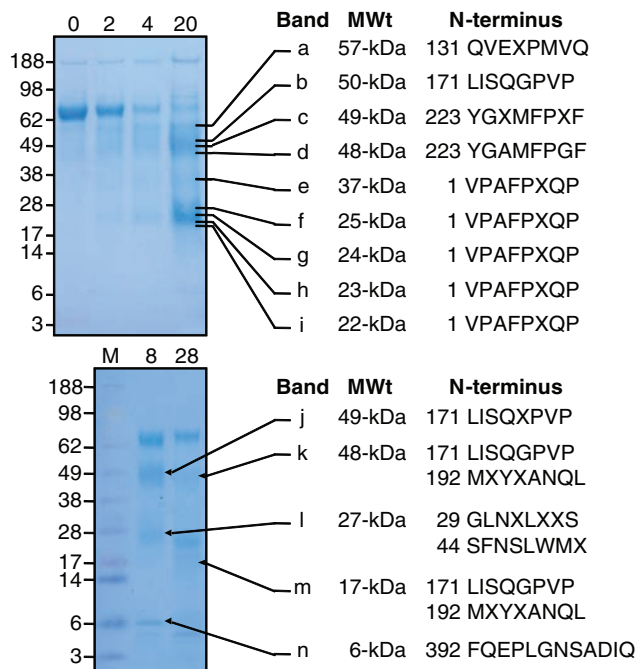


Figure 2. N-terminal sequencing of rAmbn digestion products. **(Top)** The rAmbn was digested by MMP-20 for 0, 2, 4, and 20 hrs, and samples from these digests were separated by SDS-PAGE and stained with CBB, or transferred to a membrane, which was lightly stained with CBB, and 9 bands (a through i) were excised. The apparent molecular weight and N-terminal sequence of each band are indicated. Bands f, h, and i contained the sequences indicated, starting with Val¹, as well as sequences starting with Ala³. It is not known if MMP-20 makes the cleavage before Ala³ or if the signal peptidase alternatively cleaves after Val¹ and Ala³. **(Bottom)** The rAmbn was digested by Klk4 for 8 and 28 hrs, and samples from these digests were separated by SDS-PAGE and stained with CBB, or transferred to a membrane, which was lightly stained with CBB, and 5 bands (j through n) were excised. The N-terminal sequences of the bands are indicated. Bands k, l, and m gave 2 sequences.

Klk4 and rMMP-20 were incubated with 6 FRET-Ambn synthetic peptides that covered the cleavage sites best characterized from *in vivo* studies. The products were analyzed by HPLC and by mass spectroscopy (Fig. 3). The HPLC profiles show that both enzymes were able to digest all of the peptides, with the lone exception that Klk4 did not digest the 15-kDa peptide centering on Gln¹³¹. Mass spectrometry identified the sites cleaved within the peptides. MMP-20 cleaved all 6 peptides at the exact sites that have been observed on ameloblastin cleavage products isolated *in vivo*. Klk4 cleaved at other sites, with the lone exception that Klk4 cleaved on the N-terminal side of Leu¹⁷¹ on the 17-kDa peptide. A summary of these findings is shown in Fig. 4.

DISCUSSION

We identified the cleavage site that generates the N-terminus of the 23-kDa porcine ameloblastin cleavage product, and have developed a stable cell line that expresses and secretes full-length, glycosylated ameloblastin. N-terminal sequencing

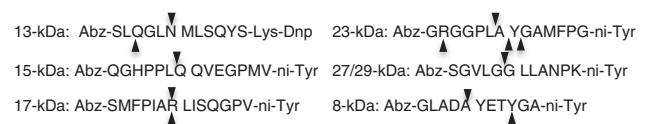


Figure 3. FRET peptide cleavage sites identified by mass spectrometry. Arrowheads above each peptide sequence indicate sites cleaved by MMP-20. Arrowheads below each peptide sequence indicate sites cleaved by Klk4. MMP-20 cleaved each peptide exactly at the sites corresponding to ameloblastin cleavages catalyzed *in vivo*: on the N-terminal sides of Met³², Gln¹³¹, Leu¹⁷¹, Tyr²²³, Leu³⁰¹, and Tyr³⁴³.

of rAmbn cleavage products generated by MMP-20 demonstrated that MMP-20 cleaves rAmbn at the same sites as have been shown to occur *in vivo*. Mass spectrometric analyses of 6 FRET peptides digested by MMP-20 and Klk4 showed that MMP-20 specifically catalyzed all of the cleavages that have been identified by the characterization of ameloblastin cleavage products isolated from developing teeth, and that Klk4 can catalyze only one of these cleavages. This bolsters the conclusion that MMP-20, but not Klk4, catalyzes the processing of enamel proteins during the secretory stage of amelogenesis.

Recombinant MMP-20 cleaved rAmbn starting on the N-terminal side of the protein, so that the N-terminal cleavage products are smaller than the C-terminal cleavage products, which mimics the *in vivo* pattern (Murakami *et al.*, 1997; Uchida *et al.*, 1997). Based upon what can be deduced from *in vivo* and *in vitro* analyses, we propose that Ambn³⁹⁵ is digested by MMP-20 initially at one of three sites—after Gln¹³⁰, Arg¹⁷⁰, and Ala²²²—generating 6 cleavage products. These initial products are then cleaved a second or third time at these same sites, as well as at secondary sites that are located mostly near the C-terminus, after Gly³⁰⁰, Arg³¹⁹, Ala³⁴², and Asn³¹. The end result is that N-terminal ameloblastin cleavage products (13-, 15-, and 17-kDa) accumulate in the sheath space throughout the enamel layer (Uchida *et al.*, 1991), while calcium-binding ameloblastin C-terminal cleavage products (13- and 15-kDa) (Yamakoshi *et al.*, 2001) are in the rods and are not detectable beyond a depth of 50 μ m (Murakami *et al.*, 1997). The cleavage pattern is consistent with an *ab initio* 3D model of human ameloblastin that portrays ameloblastin as having N- and C-terminal domains that are connected by an unstructured linker that is susceptible to degradation (Vymetal *et al.*, 2008).

The functions of secreted ameloblastin and its MMP-20 cleavage products are not known. Some reports suggest that ameloblastin is important for cell adhesion (Cerny *et al.*, 1996; Fukumoto *et al.*, 2004), possibly by binding heparin (Sonoda *et al.*, 2009) or fibronectin (Beyeler *et al.*, 2010). The evidence, however, is not strong. In the ameloblastin mutant mice, the enamel surface is so pathological that cell attachment to it might fail even if ameloblastin was not part of the attachment apparatus. Heparin and fibronectin have not been localized along the distal membrane of secretory-stage ameloblasts, and fibronectin was not among the secreted and membrane proteins identified in the rat incisor enamel organ by signal-trap screening (Moffatt *et al.*, 2006). The putative ameloblastin cell-binding motifs (VTKG and KRH-rich motifs for heparin and VPIMDFADPQFPT for fibronectin) are not well-conserved among species. Glycosylated

rAmbn showed only weak cell adhesion properties equal to those of amelogenin (Zeichner-David *et al.*, 2006). Others have noted that ameloblastin has growth-factor-like properties (Fukae *et al.*, 2006; Zeichner-David *et al.*, 2006). Future studies with glycosylated ameloblastin isolated from stable cell lines may help clarify the function of ameloblastin.

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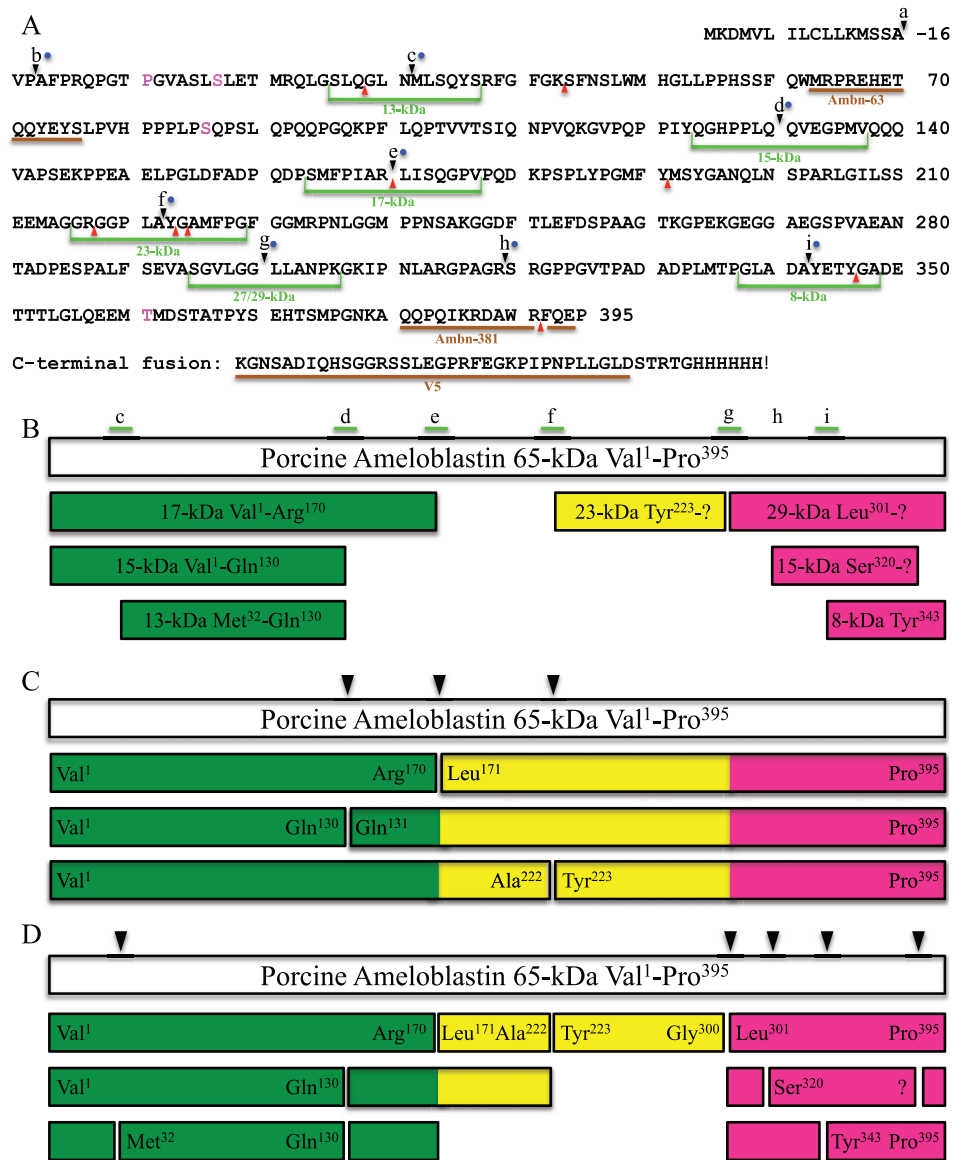


Figure 4. Ambn³⁹⁵ sequence and cleavage sites. **(A)** Sites where ameloblastin is cleaved *in vivo* are lettered: (a) Val¹, signal peptidase cleavage site (Uchida *et al.*, 1995); (b) Ala³, possible alternative signal peptide cleavage site or MMP-20 site (Iwata *et al.*, 2007); (c) Met³¹, N-terminus of the 13-kDa (Hu *et al.*, 1997); (d) Gln¹³¹, C-terminus of the 13- and 15-kDa (Hu *et al.*, 1997); (e) Leu¹⁷¹, C-terminus of the 17-kDa (Fukae *et al.*, 2006); (f) Tyr²²³ (this report); (g) Leu³⁰¹, N-terminus of 27- and 29-kDa calcium-binding proteins (Murakami *et al.*, 1997); (h) N-terminus of the 13- and 15-kDa calcium-binding proteins (Yamakoshi *et al.*, 2001); and (i) N-terminus of an 8-kDa peptide (Yamakoshi *et al.*, 2006a). Green lines indicate synthetic peptides used in this study. Blue dots indicate sites shown to be cleaved by MMP-20 *in vitro*. Red arrowheads indicate sites shown to be cleaved by Klk4 *in vitro*. Brown lines indicate antigen-binding sites for antibodies used in this study. **(B)** Porcine ameloblastin cleavage products that have been isolated and characterized *in vivo*. White bar shows the porcine ameloblastin 65-kDa protein; colored bars are cleavage products. Colors in bars indicate the N-terminal region (Val¹ to Arg¹⁷⁰; green), middle (Leu¹⁷¹ to Gly³⁰⁰; yellow), and C-terminal region (Leu³⁰¹ to Pro³⁹⁵; mauve). **(C)** Initial cleavage products based upon the digestion of rAmbn by MMP-20 *in vitro*. Arrowheads indicate initial cleavage sites. **(D)** Secondary MMP-20 cleavages (arrowheads) continue the processing of ameloblastin to yield additional cleavage products. Labeled ends of bars have been confirmed experimentally; unlabeled bars are generated by multiple MMP-20 cleavages, but have not been confirmed experimentally.

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