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A Novel Matrix Metalloprotease-like Enzyme (Karilysin) of the Periodontal Pathogen *Tannerella forsythia* ATCC 43037

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Abstract

Proteases of *Tannerella forsythia*, a pathogen associated with periodontal disease, are implicated as virulence factors. Here, we characterized a matrix metalloprotease (MMP)-like enzyme of *T. forsythia* referred to as karilysin. Full length (without a signal peptide) recombinant karilysin (49.9 kDa) processed itself into the mature 18 kDa enzyme through a sequential autoproteolytic cleavages both at N- and C-terminal profragments. The first cleavage at the Asn14-Tyr15 peptide bond generated the fully active enzyme (47.9 kDa) and subsequent truncations at the C-termini did not affect proteolytic activity. Mutation of Tyr15 to Ala generated a prokarilysin variant that processed itself into the final 18 kDa form with greatly reduced kinetics. Inactive prokarilysin with the mutated catalytic Glu residue (E136A) was processed by active karilysin at the same sites as the active enzymes. The karilysin proteolytic activity and autoprocessing were inhibited by 1,10-phenanthroline and EDTA. Calcium ions were found to be important for both activity and thermal stability of karilysin. Using the CLiPS technology, specificity of karilysin was found to be similar to that of MMPs with preference for Leu/Tyr/Met at P1' and Pro/Ala at P3. This specificity and the ability to degrade elastin, fibrinogen and fibronectin may contribute to the pathogenicity of periodontitis.

Keywords

metalloprotease; pathogenicity; periodontitis; protease specificity; protein expression; virulence factor

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Introduction

Arguably, periodontitis is the most prevalent infection-driven chronic inflammatory disease of humankind (Cobb et al., 2009). Progression of the disease is manifested by alveolar bone resorption and the loss of attachment between the tooth and the gingival tissues resulting in the formation of deep periodontal pockets. In severe cases, the disease can lead to loss of the dentition. Further, a growing body of evidence implicates periodontitis as an important factor in development of cardiovascular diseases and rheumatoid arthritis (de Pablo et al., 2009, Erridge, 2009; Wegner et al., 2009).

Although more than 700 microbial taxa have been identified in the human oral cavity, only a handful of bacterial species was found associated with development and/or progression of periodontitis. Among those bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia* (formerly *Bacteroides forsythus*) and *Treponema denticola* are referred to as the “red complex” and are considered to be the major periodontopathogens (Socransky et al., 1998). A common feature of these pathogens is production of high levels of proteolytic activity, especially with “trypsin-like” activity. Before the advent of molecular biology based diagnostic techniques, detection of a trypsin-like activity using benzoyl-arginyl- β -naphthyl amide in the fluid collected from a crevice between a tooth and the gingiva (gingival crevicular fluid) was used to detect the red complex bacteria (Loesche et al., 1990; Seida et al., 1992). Up to date, proteases with the trypsin-like activity from *P. gingivalis* and *T. denticola* have been characterized and their role as virulence factors is fairly well documented (Potempa et al., 2000; Eley and Cox, 2003; Potempa & Pike, 2009). Especially there is a vast knowledge about the pathological role of gingipains, cysteine proteases of *P. gingivalis*, which play key role in nutrients and growth factor acquisition (Smalley et al., 2008), in evasion of host defense mechanisms (Carlisle et al., 2009; Potempa et al., 2009; Puklo et al., 2009) and have potential to derail regulation of inflammatory reaction (Guzik et al., 2007; Fitzpatrick et al., 2009; Kantyka et al., 2009).

In stark contrast to other two “red complex” organisms, literally nothing is known about proteases of *T. forsythia*. Up to date, only one proteolytic enzyme referred to as PrtH was partially characterized as a recombinant protein (Saito et al., 1997). Based on protease inhibitor analysis, PrtH was suggested to be a cysteine protease which has been recently found to be identical to a protein named Forsythia Detaching Factor (FDF) (Nakajama et al., 2006). Interestingly, bioinformatic analysis suggests that PrtH is remotely related to caspases and other proteases with a caspase-like fold, such as gingipains (Pei and Grishin, 2009). In addition to PrtH, *T. forsythia* potentially can secrete several other proteases since several genes encoding putative secretory proteases (with a signal peptide) of serine-, cysteine- and metallo-peptidase catalytic classes are present in genome of this bacterium. To verify this hypothesis, we hereby describe and characterize the product of a gene encoding a MMP-like protease (Los Alamos Oral Pathogens Database Accession No. TF0367).

Results

Cloning and sequencing of a gene encoding karilysin (TF0367)

Analysis of the sequenced genome of *T. forsythia* ATCC 43037 (<http://www.oralgen.lanl.gov/>) revealed a cluster of ORFs encoding putative proteases, among others, a metalloprotease with high degree of identity (in the range from 46% to 53%) to a peptidase domain of matrix metallopeptidases (family M10A), especially MMP-13. The translated coding sequence of TF0367 lacked a signal peptide and seemed to be truncated in comparison to the MMP-13 catalytic domain. Therefore, the 5' region to the open-reading-frame (ORF) of TF0367 was resequenced to reveal an extraneous thymidine at position 149 of the gene, resulting in a frameshift event. The absence of this base in the corrected sequence resulted in an ORF extension of an additional 57 residues at the amino terminus of the translated

product of TF0367 to include a signal peptide (Figure 1A). The corrected sequence of the entire gene excluding the signal peptide was cloned into the expression vector (pGEX-6-P1). Further, DNA sequencing of the entire construct revealed another region of major discrepancy within the gene, presumably from incorrect genome shotgun sequence assembly of homologous regions with other proteases (Figure 1B). After these corrections, it becomes apparent that the TF0367 gene encodes a secretory protein consisting of 472 amino acid residues including the signal peptide (Genbank Accession No. GQ856797). Sequence alignment with MMPs suggested a presence of a short N-terminal profragment, followed by an MMP-like peptidase domain (Figure 2) and ending with a unique C-terminal extension present exclusively in other putative secretory proteases of *T. forsythia*.

Karilysin expression and purification

Wild-type prokarilysin and the protease mutants (proKly-Y15A and proKly-E136A) were overexpressed as 76 kDa fusion proteins. Both the fusion proteins as well as tag-free prokarilysins (50 kDa) were purified to homogeneity after the N-terminal GST molecule was removed by digestion with the PreScission protease (Figure 3). The N-terminal sequence analysis of the tag-free karilysin revealed the NH₂-Gly-Pro-Leu-Gly-Ser-Gln-Arg-Leu-Tyr-Asp-Asn-Gly-Pro-Leu-Tyr- sequence with first four residues derived from the vector as expected after the cleavage of the fusion protein with PreScission protease. The molecular mass of the recombinant protein was determined to be 50,052 Da by MALDI-TOF mass spectroscopy in correspondence to the molecular mass of NH₂-Gly-Pro-Leu-Gly-Ser-preKly (49,944 Da), calculated based on the amino acid composition inferred from the corrected DNA sequence of the TF0367 gene.

Activation and autoprocessing of GST-proKly, proKly and proKly^{Y15A}

Freshly purified GST-proKly has low proteolytic activity which increased up to 5-fold in a time-dependent manner when the purified proteins were incubated at either 21°C or 37°C (Figure 4A). Although the rate of activation was considerably faster at the higher temperature, a gradual increase in proteolytic activity also was observed during the prolonged storage of the enzymes on ice (0°C) (data not shown). Significantly, the released activity was stable both at 21°C and 37°C for several days (Figure 4B, C). This results suggested that karilysin autoactivates itself and therefore we next analyzed if this process is accompanied by autoprocessing of the nascent protein. Indeed as expected, SDS-PAGE analysis of samples of proKly (Figure 5A) and proKly^{Y15A} (Figure 5B) incubated for different time intervals revealed a progressive time-dependent processing of the initial protein with concomitant formation of discrete transient products and final accumulation of the 18 kDa enzyme. As in the case of activity enhancement, the processing occurred faster at higher temperature (Figure 5C).

N-terminal sequencing of individual bands complemented by MALDI-TOF analysis of selected bands revealed a sequence of proteolytic events in karilysin processing and allowed identification of stable intermediates (Figure 5). Since the initial cleavage apparently occurred at Asn14-Tyr15 peptide bond, we proceed to analyze processing of a proKly^{Y15A} mutant protein. In comparison to the wild-type enzyme, the mutated karilysin processed itself at a much slower rate and even after 48h of incubation the unprocessed protein was still present in the sample (Figure 5B). The slow processing of proKly^{Y15A} correlates very well with the slow rate of the release of the proteolytic activity from the mutated zymogen (Figure 5C).

Zymography analysis of autoprocessed products of GST-proKly and proKly revealed proteolytically active forms of the enzyme corresponding to Kly-derived fragments of 38 kDa (Kly38) and 18 kDa (Kly18) (Figure 6B). Since the former fragment, although transient, shows significant stability (Figure 5A) and the latter constitutes the final stable form of karilysin, it

was possible to purify these forms of the enzyme using gel filtration chromatography (Figure 6A).

To show that processing of proKly is not due to contamination with *E. coli* proteases, we investigated the stability of an inactive prokarilysin (E136A) mutant. As shown in Figure 7, even after 48 h incubation, there was no trace of degradation of the mutated protein. However, when active Kly38 was added in a catalytic amount, progressive processing of proKly^{E136A} occurred leading to formation of the 18 kDa stable product apparently identical to Kly18 released from wild-type proKly or proKly^{Y15A} (compare Figure 5 and 7). The autoprocessing of wt-proKly as well as the conversion of proKly^{E136A} into Kly18^{E136A} exerted by active Kly was completely inhibited by EDTA and 1,10-phenanthroline (data not shown) further arguing for autocatalytic mechanism of karilysin maturation.

Proteolytic activity

Freshly purified full length karilysin (proKly) is apparently latent but within 30 min at 37°C, it is fully converted into the N-terminally truncated form of the enzyme (Kly48) due to autocatalytic cleavage of the Asn14-Tyr15 peptide bond. Sequentially, a second cleavage releases the most C-terminal domain and a relatively stable Kly38 is formed. The correlation between time-dependent formation of Kly48 and Kly38 and an increase of proteolytic activity argues that Kly48 is already proteolytically active (Figure 5). Unfortunately, the transient character of Kly48 makes it impossible to enzymologically characterize this form of karilysin.

The direct comparison of the Kly38 and Kly18 activity on casein, 93.5 RFU/sec and 48.7 RFU/sec, respectively, indicates that the higher molecular mass enzyme is approximately twice as active as the low molecular mass karilysin. Both forms of karilysin also cleaved gelatin and elastin but at a 2- and 3-fold lower rate, respectively, than that for casein degradation. Also on these substrates, Kly38 showed higher activity than Kly18. Further, Kly38 was found to efficiently degrade fibrinogen and fibronectin. At a 100:1 (substrate:enzyme) molar ratio, about 50% of fibronectin was truncated within 30 min and the native protein disappeared after 2 h (Figure 8). In the case of fibrinogen, the α -chain was degraded within short time of incubation with Kly38 (Figure 8). As expected for metalloproteases, cleavage of both substrates was totally inhibited by 1,10-phenanthroline.

Inhibition profile, stability and pH optimum

Table 1 shows the inhibition of Kly38 by common protease inhibitors. The lack of inhibition by inhibitors other than Zn²⁺ chelating agents (EDTA, 1,10-phenanthroline) confirms that karilysin is a typical metalloprotease. Interestingly, enzyme activity was considerably enhanced by Ca²⁺ but inhibited by an excess of Zn²⁺. Calcium was also shown to significantly enhance the maturation processing of proKly to Kly38 without affecting the sequence and the pattern of formation of transient processing products (data not shown). The effect of calcium is most likely due to stabilization of the protein structure. While in the presence of CaCl₂, the enzyme was fully stable up to 40 min at 70°C, karilysin incubated without calcium lost 50% activity within 30 min (Figure 9A).

The pH optimum of Kly38 for the casein degradation was found at pH 8.0 but activity was dependent on buffer composition. In Tris at this pH, the karilysin activity was 2-fold higher than in HEPES at the same pH (Figure 9B). This difference in activity may reflect differences in ionic strength exerted by the buffering species themselves. Nevertheless, it is likely that in physiological conditions, the enzyme is active in the broad pH range from pH 6.5 to 8.5.

Enzyme specificity

Analysis of the autoproteolytic cleavage sites during the proKly maturation process revealed that the protease has strong preference to hydrolyze specifically peptide bonds on the NH₂-terminal side of hydrophobic, both aliphatic and aromatic residues (the P1' site) (Table 2).

To map the subsite specificity of karylysin in detail, we employed a cellular library of peptide substrates (CLiPS) (Boulware and Daugherty, 2006; Rice and Daugherty, 2008). Using the CLiPS library, including eight consecutive substrate positions, each randomized with 20 natural amino acids, we fully confirmed exclusive preference of karylysin for hydrophobic residues, especially Leu, Tyr and Met at the P1' position. Interestingly, the enzyme also showed very high selectivity for Pro or Ala at P3. Together, the preferential cleavage at the Xaa-Pro-Xaa-Xaa#Leu-Xaa (where “#” and “Xaa” designates the hydrolyzed peptide bond and any amino acid residue, respectively) resembles specificity of MMPs, especially, MMP-13.

Discussion

The majority of proteases are synthesized as zymogens that await activation at a suitable time to protect the biosynthetic machinery of the cell against premature activation and to act as a timing event in biological function (Neurath, 1991). Thus, one of the key events in any proteolytic pathway is the conversion of the zymogen to the active protease. The primary structure of numerous secretory proteases encoded in the *T. forsythia* genome bearing N-terminal profragments and unique C-terminal extensions suggests such a mechanism of control is present in the organism. Furthermore, both N- and C-terminal extensions of these proteases may possess additional biological functions. Therefore, this study aimed to produce the precursor form of an apparent secretory metalloprotease of *T. forsythia*, to investigate its processing and to characterize the proteolytic activity of the active forms of the enzyme.

The initial translation product deduced from the corrected karilysin gene sequence is a precursor protein with a 20-residue signal peptide, a 14-residue N-terminal pro-peptide, a 161-residue MMP-like domain and a 277-residue C-terminal region. The recombinant form of karilysin expressed without the signal peptide but containing five vector-derived residues (GPLGS) or the GST molecule preceding the native N-terminus is apparently a zymogen (proKly). Maturation of proKly occurs through the sequential appearance of intermediates leading to the ultimate product (Figure 10). The first autoproteolytic cleavage at the Asn14-Tyr15 peptide bond is essential for activation and further processing since mutation of this site (Tyr15Ala) significantly hinders generation of active enzyme and authentic downstream intermediates. In the wild-type karilysin, the second processing step at Pro371-Phe372 removes 86 C-terminal residues generating the 38 kDa form of the active karilysin (Kly38). Finally, the third processing step at Leu180-Tyr181 removes the remainder of the C-terminal extension yielding the mature enzyme of 18 kDa (Kly18). Since Kly38 was found to be more active than Kly18 and the processing rate at this step was slow, it raised the question whether Kly38 is the physiologically relevant entity. This may be the case but a multiple alignment with MMP-13 from mammalian species revealed that the mature MMP-13s have high homology only to the Kly18 region, arguing for this form of karilysin to be the mature form of the enzyme (Figure 2).

In the case of proKly^{Y15A}, the sequence of cleavages was disturbed and before the N-terminus was truncated, the karilysin forms (proKly38^{Y15A} and proKly18^{Y15A}) without the C-terminal domain were generated.

Interestingly, all of the main cleavages in proKly processing occurred proximal to putative C-terminal domains with sequences which were strongly conserved in different proteases of *T. forsythia* (Figure 10). The transient accumulation of these processed non-proteolytic fragments

of karilysin could be detected and possible biological function of these fragments will require separate studies. At present, however, it is tempting to speculate that the conserved C-terminal domain participates in secretion of *T. forsythia* proteases as Kly18 is fully active yet missing this region. This is analogous to that of the gingipain RgpB of *P. gingivalis* where the C-terminus of karilysin contains a motif (KXXXXK) that has been described to be essential for RgpB maturation and secretion (Nguyen et al., 2007). As *T. forsythia* possess homologues of novel proteins that have been shown to participate in gingipain secretion by *P. gingivalis* (Sato et al., 2005; Saiki and Konishi, 2007; Ishiguro et al., 2009; Nguyen et al., 2009), it is likely that karilysin is exported by the same mechanism. Further, despite belonging to different catalytic classes, the precursors of both enzymes seems to have at least a small degree of proteolytic activity to be able to undergo sequential auto-proteolytic processing at both N- and C-termini (Mikolajczyk et al., 2003).

Alignment of Kly18 sequence with MMPs clearly indicates that karilysin is closely related to MMPs (metzincins), the subfamily of metalloproteases that are characterized by 3-histidine zinc-binding motif (HEXXHXXGXXH) and a conserved methionine turn following the active site (Bode et al., 1993). The prodomain of a typical MMP is about 80 amino acids and contains the consensus sequence PRCXXPD. The cysteine-thiol and zinc ion interaction keeps proMMPs in a latent state and zymogens activation occurs by disruption of this interaction (the so called “cysteine switch” (Springman et al., 1990)) as a result of (i) direct cleavage of the pro-domain; or (ii) reduction of the free thiol; or (iii) allosteric perturbation of zymogen (Ra and Parks, 2007). Thiol reduction and allosteric controls would ultimately lead to inter- or intra-molecular autolytic cleavage of the prodomain: the final step in MMPs activation. The karilysin prodomain is very short (14 residues) and lacks a cysteine residue. Nevertheless, the propeptide exerts enzyme latency using an apparently unique mechanism sensitive to structural perturbation by SDS as proKly, alike to proMMPs, is fully active in substrate zymography (Figure 6).

Application of the robust CLiPS methodology to map specificity of substrate binding subsites revealed a strong preference of the enzyme for Leu then Tyr and Met residue at P1' and Pro or Ala at P3 site of the substrate (Table 2). This resembles specificity of MMPs, showing similar preference for cleaving at the Pro/Ala-Xaa-Xaa # Leu/Tyr consensus sequence (where # indicates a scissile peptide bond) (Tallant et al., 2009). Interestingly, the preference for Pro/Ala at P3 is not fully conserved at the autocatalytic cleavage sites. With an exception of one cleavage during the proKly^{Y15A} autoprocessing, other cleavages also occurred preferentially at the Xaa#Hyd-Xaa-Leu consensus sequence, where Hyd indicates hydrophobic aliphatic or aromatic residue.

Karilysin has broad substrate specificity and degrades several proteins including casein, gelatin, fibrinogen, fibronectin and elastin at pH ranging from neutral to slightly alkaline. Degradation of the latter three proteins may have implications in *T. forsythia* pathogenicity. The enzyme structure is stabilized by Ca²⁺ which also enhances karilysin proteolytic activity and accelerates the rate of autoprocessing. However unlike ulilysin, a prototype of a new family of MMPs which includes prokaryote metzincins (Tallant et al., 2006), calcium does not mediate the autolytic activation of proKly *per se*.

Pathogenic bacteria metalloproteases are recognized as important virulence factors. The same may apply for karilysin. The presence of karilysin gene and its transcript is detected in gingival crevicular fluid at periodontitis sites infected with *T. forsythia* (Sigrun Eick, personal communications) indicating that karilysin is expressed *in vivo*. Despite that, karilysin was not among 221 proteins identified in the proteome of the *T. forsythia* cell envelope (Veith et al., 2009) arguing that the protein may be secreted or is expressed in a significant amount only under special conditions such as during host colonization. Therefore defining the karilysin

pathological role will require delineation of the spectrum of physiological substrates using robust techniques (Van Damme et al., 2008). This future study should reveal the contribution of karilysin towards *T. forsythia* pathogenicity.

Materials and methods

Chemicals and reagents

Restriction endonuclease *Bam*HI and *Xho*II, T4 DNA ligase, and dNTP were purchased from Fermentas (Burlington, ON, Canada). DNA polymerase and Gel Extraction Kit were obtained from Finnzyme (Woburn, MA, USA) and QIAGEN (Valencia, CA, USA), respectively. DNA clean up system and plasmid extraction kits were purchased from A&A Biotechnology (Gdynia, Poland). Site-directed mutagenesis kit was from Stratagene (La Jolla, CA, USA). Expression vector pGEX6p-1, Glutathione-Sepharose 4 Fast Flow and the 3C protease (PreScission) were purchased from Amersham Bioscience (Buckinghamshire, UK). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Billerica, MA, USA). Fluorescent-labeled protease substrates, including FITC-Casein, DQ-gelatin and DQ-elastin were from Molecular Probes (Carlsbad, CA, USA). Human fibrinogen, fibronectin, bovine serum albumin (BSA) and all other chemicals were purchased from Sigma (St. Louis, MO, USA).

Expression and mutant construction

Genomic DNA of *T. forsythia* was extracted from strain ATCC 43037. The entire karilysin (*kly*) gene (TF0367/TF0368; <http://www.oralgen.lanl.gov>), except for the nucleotide sequence that encodes the signal peptide, was amplified by PCR by using forward primer, (Kly-F) 5'-AAGGGATCCCAGCGCCTATACGATAATGG-3' with an *Bam*HI recognition site (underlined) and reverse primer (Kly-R) 5'-CCGCTCGAGTTACTTTTTGATCAACTTCTGCG-3' with an *Xho*I recognition site (underlined). The PCR product was purified and cloned into the *Bam*HI/*Xho*I site of pGEX-6P-1 expression vector, which provides the coding sequence for an N-terminal glutathione-S-transferase (GST). This genetic manipulation inserts five residues (Gly-Pro-Leu-Gly-Ser) before the N-terminal glutamine residue of karilysin after the GST moiety is removed by cleavage with the PreScission protease cleavage. The recombinant plasmid (pGEX-6P1-Kly) was transformed into *Escherichia coli* strain BL21(DE3) under the control of the T7 promoter. The wild-type construct was used to produce Tyr15Ala (Y15A) and Glu136Ala (E136A) mutations using overlap extension PCR (Higuchi et al., 1988) by following the protocol provided by the manufacturer (Stratagene, La Jolla, CA, USA) of the QuikChange Site-Directed Mutagenesis Kit. Briefly, Y15A and E136A mutants were produced by replacing the TAT and GAG codons, respectively for Tyr15 and Glu136 with the GCT codon for Ala using the following sets of mutagenic primers: Y15A-F, 5'-CAGGGGACAATAACGCTGTTCTTCAAGGTTCAAATGG-3' and Y15A-R 5'-CCATTTTGAACCTTGAAGAACAGCGTTATTGTCCCCTG-3' and E136A-F 5'-CAGTTGCGGCACATGCTATCGGTCATCTATTAGG-3' and E136A-R 5'-CCTAATAGATGACCGATAGCATGTGCCGCAACTG-3'. The mutated constructs were verified by DNA sequencing.

Resequencing of TF0367 and TF0368 loci of *T. forsythia*

The region encompassing the TF0368 gene and 5' region of TF0367 gene were amplified by PCR (primers: TF0368SacI 5'-TAGAGCTCGTTTTACGCTTTGGTTGG and TF0367Hind3R 5'-CTCGAAGCTTACCTGTATTTCCATCAAAAGGA) using Accuprime Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and inserted into pUC19 at SacI and HindIII sites. The clones were sent away for DNA sequencing using the TF0367Hind3R primer.

Recombinant karilysin expression and purification

Protein production and purification were essentially the same for the wild-type and mutant proteins. Routinely, cells freshly transformed with expression plasmids were grown at 37°C in 1 liter LB medium to $A_{600} = 0.5$ and cooled down to 21°C for 30 min. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) was added into the culture (final concentration 0.3 mM), and the culture was further incubated at 21°C for 3 h to induce protein production. Cells were harvested, washed with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , pH 7.4) and resuspended in 50 ml of PBS. Cells were lysed by ultrasonication on ice for about 5 minutes. The cell lysates were cleared by centrifugation ($12,000 \times g$), filtered through 0.45 μm membrane and applied onto a glutathione-Sepharose column (2 ml bed volume) equilibrated with PBS at 1 ml/min flow rate. The column was then washed with 150 ml PBS to elute the unbound proteins. The recombinant karilysin was then eluted in 10 ml of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM glutathione). Alternatively, 5 ml of PBS containing 100 μl of Prescission protease stock solution (1 U/ μl) was applied onto the column and incubated overnight at 4°C. Recombinant karilysin was eluted with 5 ml PBS and concentrated by ultrafiltration using Vivaspin concentrator (VIVA). Final purification of karilysin was accomplished by gel filtration on Superdex 75 (16/60 gel filtration column) pre-equilibrated with 50mM Tris-HCl, pH 8.0.

Individual and pooled fractions were analyzed by SDS-PAGE. The total amount of proteins recovered at each step of the purification procedure was determined by bicinchonic acid (BCA) assay using bovine serum albumin as a standard. The tag-free karilysin forms were routinely purified with the yield of circa 5 mg per liter of culture.

Gel electrophoresis and zymography

Karilysin purification and autocatalytic processing as well as protein substrates degradation by karilysin was monitored by SDS-PAGE using 10% gels and the Tris-HCl/Tricine buffer system (Schagger and von Jagow, 1987). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 10% acetic acid and destained.

Zymography analysis was performed on samples solubilized in SDS buffer (4% SDS, 20% glycerol, 0.125 M Tris-HCl, pH 6.8) for 30 min at 37°C and electrophoresed on 12% SDS-PAGE with gelatin or casein incorporated into the gel at 0.1 mg/ml. Gels were washed with 2.5% Triton X-100, followed with 0.2 M Tris-HCl, pH 7.8 containing 5 mM CaCl_2 and incubated in this buffer for 3 h at 37°C. Finally, the gel was then stained with 0.1% Amido black in methanol-water-acetic acid (3:6:1) for 1 h and destained with the same solution without the dye, which revealed clear zones of substrate hydrolysis on a blue background.

N-terminal sequence analysis

Wild-type karilysin and the Y15A and E136A mutant proteins, as well as their truncated variants generated during autoprocessing were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Protein bands were visualized by Coomassie Brilliant Blue staining, excised and analyzed by automated Edman degradation using a Procise 494HT amino acid sequencer (Applied Biosystems, Foster City, CA, USA).

MALDI-TOF analysis

Protein sample analysis was performed on a Voyager-DETM STR (Applied Biosystems, Foster City, CA, USA) controlled by the Data Explorer software (versions 3.4.0.0. and 4.4., Applied Biosystems). Samples were concentrated by microreverse phase (ZipTip C4, Millipore, Billerica, MA, USA) and eluted onto the target using a 20 $\mu\text{g}/\mu\text{l}$ solution of Sinapic acid (70% acetonitrile, 0.1% TFA).

Proteolytic processing and activation of karilysin

GST-proKly, wt-proKly, proKly^{Y15A} and proKly^{E136A} in 0.1 M Tris-HCl, pH 8.0 were incubated at different temperatures (4°C, 21°C and 37°C) for up to 36 h. At specific time-points, aliquots were withdrawn for proteolytic activity measurement and SDS-PAGE analysis. In the case of proKly^{E136A} the time-dependent proteolytic processing at 37°C of this inactive variant of karilysin by the purified active enzyme at a substrate/enzyme ratio 100:1 was also determined by SDS-PAGE analysis. ProKly^{E136A} incubated alone for 48h served as a control.

Proteolytic activity assay and karilysin characterization

Protease activity was detected using various fluorescent substrates including FITC-casein, DQ-gelatin and DQ-elastin. Routinely assays were performed at 37°C using 500 nM of enzyme in 100 mM Tris-HCl, 5mM CaCl₂, pH 8.0, at substrate concentrations of 25µg/ml. Released fluorescence was measured using micro-titer plate reader (Spectra Max Gemini, Molecular Devices) at excitation/emission wavelengths of 485/538 nm for FITC-casein and 500/520 nm for DQ-gelatin and DQ-elastin.

Human plasma fibrinogen and fibronectin were incubated with Kly at a substrate/enzyme 100:1 weight ratio up to 24 h at 37°C in 100 mM Tris-HCl, 5 mM CaCl₂, pH 8.0, and samples withdrawn at specific time points were subjected to SDS-PAGE. Proteins incubated alone served as controls.

FITC-casein was used as a substrate to determine pH optimum, the enzyme thermal stability, and effect of inhibitors and divalent metal ions on Kly proteolytic activity. The pH optimum was assayed using the following buffers at 100 mM concentration: MES (pH 5.5, 6.0, and 6.5), MOPS (pH 6.5, 7.0, and 7.5), HEPES (pH 7.0, 7.5, and 8.0), Tris (pH 7.5, 8.0, and 8.5), and CAPS (pH 8.5, 9.0, and 10.0). All buffers contained 5 mM CaCl₂. The effect of temperature on the activity of purified karilysin was performed in the presence (5 mM) or absence of CaCl₂ in 100 mM Tris-HCl, pH 8.0. The enzyme was incubated at 0, 4, 10, 18, 28, 37, 45, 55 and 70°C for 2 h and residual activity measured. Thermal stability was assayed by incubating karilysin at 70°C in the presence or absence of 5 mM CaCl₂ for different periods of time and then measuring the residual activity under standard conditions. To test the effect of inhibitors and metal ions on karilysin activity, the enzyme was pre-incubated with inhibitors and metal ions in 100 mM Tris-HCl, pH 8.0, for 15 min at room temperature and the residual activity determined.

Cellular library of peptide substrates (CLiPS)

A complete consensus sequence recognized and cleaved by karilysin was discerned using a CLiPS methodology as described previously (Boulware and Schagger, 2006). In brief, a library of 10⁸ clones displaying a bait polypeptide containing an SH3 domain binding motif, eight consecutive randomized amino acids linker and a streptavidin binding peptide ligand in the context of *E. coli* membrane protein (eCPX) (Rice and Schagger, 2008), was screened for proper display and karilysin hydrolysis using fluorescence activated cell sorting (FACS). Library hydrolysis was assayed in 5 mM Tris-HCl, 50 mM NaCl, 5 mM CaCl₂, pH 7.6, at 37°C for 1.5 h. Concentration of enzyme was gradually decreased from 650 nM to 65 nM at each sorting step. Clones with intact baits show red fluorescence after incubation with phycoerythrin-conjugated streptavidin (50 nM). Clones displaying substrates that were hydrolyzed were obtained by sorting the unlabeled cells from the control culture incubated with the enzyme. During next sorting step, clones with red fluorescence were isolated from culture not exposed to protease. After 9 rounds of sorting the cleavage of selected clones was verified by incubation with 124 nM karilysin for 1.5 hour and clones susceptible to hydrolysis were sequenced.

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Figure 1.

Panel A: An extraneous thymidine (highlighted) in the database sequence caused a frameshift error in translation of the protein, resulting in a truncated N-terminus along with a missed signal peptide sequence (boxed). Panel B: Alignment of translated amino acid sequence of TF0367 from the database (original) and after corrections. Zn²⁺ chelating His residues and the catalytic Glu are boxed and regions conserved in other proteases of *T. forsythia* are underlined.

MER001067_ <i>Mus musculus</i>	rkprcgvpdvGEYNVFPRTLKWSQTNLTyrivnyTPDMshsevekafrka	50
MER001079_ <i>Rattus norvegicus</i>	rkprcgvpdvGVYNVFPRTLKWSQTNLTyrivnyTPDishsevekafrka	50
MER005072_ <i>Bos taurus</i>	kkprcgvpdvGEYNVFPRTLKWSKMNLtyrivnyTPDLthsevekafrka	50
MER001411_ <i>Homo sapiens</i>	kkprcgvpdvGEYNVFPRTLKWSKMNLtyrivnyTPDMthsevekafrka	50
MER104209_ <i>Erinaceus europaeus</i>	kkprcgvpdvGEYNVFPRTLKWSKTNLTyrivnyTPDLthsevekaikka	50
Karilysin	<u>qrlydngpltdgdnYVLQGSKWnKTLKYIYNSSSHLTtTERENAIRSA</u> :: . * . * * . : ** . : . * . * * : . . . : * * : * : . *	36
MER001067_ <i>Mus musculus</i>	FKVWSDVTPLNfTRIYDG-TADIMISFGTKEHGDFYPFDGPGSGLLAHAFp	99
MER001079_ <i>Rattus norvegicus</i>	FKVWSDVTPLNfTRIHDG-TADIMISFGTKEHGDFYPFDGPGSGLLAHAFp	99
MER005072_ <i>Bos taurus</i>	FKVWSDVTPLNfTRIHNH-TADIMISFGTKEHGDFYPFDGPGSGLLAHAFp	99
MER001411_ <i>Homo sapiens</i>	FKVWSDVTPLNfTRLHDG-IADIMISFGIkeHGDFYPFDGPGSGLLAHAFp	99
MER104209_ <i>Erinaceus europaeus</i>	LKVWSDVTPLNfTRINND-TADIMISFGTKEHGDFYPFDGPGSGLLAHAFp	99
Karilysin	FALWSDKSTLSFIQVYNPNQADIKIKWEKGNHGDGYPFDGNTGILAHAFY : : *** : . * . : : : * * * * . : : * * * * * : : * * * * *	86
MER001067_ <i>Mus musculus</i>	P---GPNYGGDAHFDDDETWTSSSKGYNLFIVAAHELGHSGLDHSKDPG	146
MER001079_ <i>Rattus norvegicus</i>	P---GPNLGGDAHFDDDETWTSSSKGYNLFIVAAHELGHSGLDHSKDPG	146
MER005072_ <i>Bos taurus</i>	P---GPNYGGDAHFDDDETWTSSSKGYNLFVAAHEFGHSGLDHSKDPG	146
MER001411_ <i>Homo sapiens</i>	P---GPNYGGDAHFDDDETWTSSSKGYNLFVAAHEFGHSGLDHSKDPG	146
MER104209_ <i>Erinaceus europaeus</i>	P---GPNYGGDAHFDDDETWTSSSKGYNLFVAAHEFGHSGLGLEHSKDPG	146
Karilysin	PPPAGGNYAGHLHFDDDENWSINGSGIDLITVAAHEIGHLLGIEHSNVSS * * * . * . * * * * . * : . . . * : * : * * * : * * * : * : . .	136
MER001067_ <i>Mus musculus</i>	ALMFPIYTYTGKSHFMLPDDDVQGIQFLYGPGEDEPNPKHPKTPE	181
MER001079_ <i>Rattus norvegicus</i>	ALMFPIYTYTGKSHFMLPDDDVQGIQSLYGPGEDEPNPKHPKTPE	181
MER005072_ <i>Bos taurus</i>	ALMFPIYTYTGKSHFMLPDDDVQGIQSLYGPGEDEPNPKHPKTPE	181
MER001411_ <i>Homo sapiens</i>	ALMFPIYTYTGKSHFMLPDDDVQGIQSLYGPGEDEPNPKHPKTPE	181
MER104209_ <i>Erinaceus europaeus</i>	ALMFPIYTYTGKSHFMLPDDDVQGIQSLYGPGEDEPNPKHPKTPE	181
Karilysin	ALMPYYTGIKR---QLDNDDCLAVWDLYGYPFsISGPSSVCDQA * * * : * * * : * * *	161

Figure 2.

Alignment of karilysin and proteolytic domains of selected MMP-13. The identical residues are indicated by asterisk. Lower case sequences represent the entire profragment of karilysin and the last 10 residues of MMP profragments preceding the catalytic domains. The consensus sequence (PRCXXPD) in the profragment responsible for the cysteine switch activation of MMPs is highlighted. The conserved residues in the 3-histidine zinc-binding motif (HEXXHXXGXXH) and a conserved methionine turn following the active site characteristic for metzincins are boxed. The C-terminal extension absent in Kly38 and Kly18 is in italics. Numbering accounts for residues of catalytic domains alone. The sequence absent in TF0367 from the database is underlined.

Figure 3.

Expression and purification of GST-karilysin. *E. coli* cell extracts, the purified GST-proKly fusion protein, and tag-free proKly after digestion with PreScission were resolved by SDS-PAGE. Lane 1, molecular mass markers; lane 2, *E. coli* extract before IPTG was added; lane 3, *E. coli* extract after 3 h after protein expression stimulation with IPTG; lane 4, GST-proKly eluted from a glutathione-Sepharose; lane 5, tag-free proKly (50kDa); lane 6, proKly purified by gel filtration on a Superdex 75 column.

A)

B)

C)

Figure 4.

Time- and temperature-dependent activation and autoprocessing of GST-proKly. 20 μ M of GST-proKly was incubated at 22°C and 37°C in 0.1 M Tris-HCl, pH 8.0. At indicated time points samples were withdrawn for activity assay with FITC-casein (A) and SDS-PAGE analysis (B and C, sample incubation at 21°C and 37°C, respectively).

Figure 5. Comparison of time-dependent autoprocessing of wt-proKly (A, C) and proKly^{Y15A} (B). Both forms of karilysin at 20 μ M were incubated at 37°C in 0.1 M Tris-HCl, pH 8.0. At indicated time points samples were withdrawn for SDS-PAGE analysis (A, B) and activity assay with FITC-casein (C).

Figure 6. SDS-PAGE analysis and Casein zymogram of different purified forms of karilysin. (A) Purified forms of karilysin: GST-proKly (lane 1), wt-proKly (lane 2), Kly38 (lane 3), and Kly18 (lane 4) resolved by SDS-PAGE. LMW, molecular mass standards. (B) GST-proKly (lane 1) and samples withdrawn during the time-course of wt-proKly autoprocessing (see Fig 3) at 2 h (lane 2) and 336 h (lane 3) were subjected to casein zymography.

Figure 7.

Processing of proKly^{Glu136Ala} by wild-type karilysin. Recombinant proKly^{Glu136Ala} was incubated with Kly48 at the molar ratio of 100:1 at 37°C in 25 mM Tris-HCl, pH 8.0. At indicated time points aliquots of 5 µl were withdrawn from the reaction mixture, mixed with 5 µl of reducing SDS-PAGE sample buffer and denatured at 95°C for 5 min to stop the reaction. Samples (10 µl) were subjected to SDS-PAGE. ProKly^{Glu136Ala} and Kly38 were incubated alone at the same final concentration as in the sample and served as controls. Lane 1, LMW protein standards; lane 2, control proKly^{Glu136Ala}; lanes 3–9, proKly^{Glu136Ala} incubated with active karilysin for 0 h (lane 3), 2 h (lane 4), 4 h (lane 5), 8 h (lane 6), 12 h (lane 7), 24 h (lane 8), and 48 h (lane 9). Lane 10, Kly48 control.

Figure 8.

Degradation of fibrinogen (A) and fibronectin (B) by karilysin (Kly38). Fibrinogen (100 μ g) and fibronectin (50 μ g) were incubated at 37 °C in 100 μ l of 100 mM Tris-HCl, 5 mM CaCl_2 , pH 8.0, at the substrate/enzyme molar ratio 100:1. At indicated time points, aliquots (10 μ l) were withdrawn from the reaction mixture, mixed with 10 μ l of reducing SDS-PAGE sample buffer and denatured at 95°C for 5 min to stop the reaction. Samples (20 μ l) were subjected to SDS-PAGE. Fb and Fn incubated with karilysin in the presence of 1,10-phenanthroline (OF) served as non-digested protein control.

Figure 9.

Thermal stability and optimum pH. (A) Kly38 (0.5 mg/ml) was incubated in 0.1 M Tris-HCl, pH 8.0 in the presence or absence of CaCl₂ at 70°C. At specific time points, 10 µl aliquots were withdrawn to assay the residual enzyme activity using FITC-casein as a substrate. (B) Activity of Kly38 was determined at different pH. The activity at 0.1 M Tris-HCl, pH 8.0 was arbitrarily taken as 100%.

Figure 10.

Schematic drawing of sequential autoprocessing of proKly. The cleavage sites (solid short arrows: major primary cleavage sites, dotted arrows: secondary minor cleavage sites) were determined by N-terminal sequencing and MALDI-TOF analysis of major discrete transiently stable processed products. Fragments bearing the MMP-like protease domain (bars) and non-enzymatic fragments (double-headed arrows). Duration of discrete fragments transiently accumulated during proKly autoprocessing detected by SDS-PAGE (Figure 3) is shown on the right.

Table 1

Effect of inhibitors divalent cations on the proteolytic activity of karilysin.

Inhibitors and metal ions	Concentration	% of control
Pefablock	5 mM	97
	10 mM	100
TLCK	50 μ M	101
	100 μ M	105
Leupeptin	50 μ M	108
	100 μ M	106
E-64	50 μ M	104
	100 μ M	102
Iodoacetic acid	5 mM	105
	10 mM	109
1,10-Phenanthroline	5 mM	3
	10 mM	0
EDTA	5 mM	2
	10 mM	0
CaCl ₂	2 mM	355
	5 mM	362
	10 mM	268
ZnCl ₂	2 mM	0
	5 mM	0
	10 mM	0

Activity determined at 37°C in 100mM Tris-HCl, pH 8.0, using FITC-casein as a substrate was taken as 100%.

	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'
	g	g	A	A	A	P	L	L	Q	F	g
	g	A	S	P	M	T	Y	P	F	g	g
	q	g	g	P	A	A	Y	T	S	W	V
	g	g	P	P	A	M	Y	T	L	L	g
	g	g	A	P	A	L	Y	A	N	Y	g
	g	g	L	G	S	N	Y	S	V	Y	g
	q	g	g	P	L	L	Y	R	I	S	Q
	q	g	g	P	P	S	Y	T	L	P	S
	g	g	I	P	G	S	M	V	F	R	g
	g	P	L	A	V	N	M	T	T	g	g
	q	g	g	P	S	V	M	V	L	A	D
	M	S	S	A	M	G	M	V	g	g	s
	q	g	g	P	P	A	F	S	T	F	A
	g	R	G	P	A	N	F	S	V	g	g
	g	g	M	N	S	T	L	V	L	P	g
	g	P	R	P	A	A	V	Q	Y	g	g
consensus				P/A			L/Y/M				