7-1997

Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial

Kim A. Brogden
United States Department of Agriculture

Mark R. Ackermann
Iowa State University, mackerma@iastate.edu

Kenneth M. Huttner
Boston Children's Hospital

Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial

Abstract
Some inactive precursor proteins, or zymogens, contain small, amino terminus, homopolymeric regions of Asp that neutralize the cationic charge of the active protein during synthesis. After posttranslational cleavage, the anionic propeptide fragment may exhibit antimicrobial activity. To demonstrate this, ovine trypsinogen activation peptide, and frog (Xenopus laevis) PYL activation peptide, both containing homopolymeric regions of Asp, were synthesized and tested against previously described surfactant-associated anionic peptide. Peptides inhibited the growth of both gram-negative (MIC, 0.08 to 3.00 mM) and gram-positive (MIC, 0.94 to 2.67 mM) bacteria. Small, anionic, and charge-neutralizing propeptide fragments of zymogens form a new class of host-derived antimicrobial peptides important in innate defense.

Disciplines
Veterinary Pathology and Pathobiology

Comments
This article is from *Antimicrobial Agents and Chemotherapy* 41, no. 7 (July 1997): 1615–1617.

Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial.

K A Brogden, M Ackermann and K M Huttner
***Antimicrob. Agents Chemother.* 1997, 41(7):1615.**

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1997, p. 1615–1617 Vol. 41, No. 7
0066-4804/97/$04.00+0

Small, Anionic, and Charge-Neutralizing Propeptide Fragments of Zymogens Are Antimicrobial

KIM A. BROGDEN,[1]* MARK ACKERMANN,[2] AND KENNETH M. HUTTNER[3]

Respiratory and Neurologic Disease Research Unit, National Animal Disease Center, USDA Agricultural Research Service, Ames, Iowa 50010,[1] Department of Veterinary Pathology, Iowa State University, Ames, Iowa 50011,[2] and Joint Program in Neonatology, Department of Medicine, Boston Children's Hospital, Boston, Massachusetts 02115[3]

Received 19 December 1996/Returned for modification 21 February 1997/Accepted 24 April 1997

Some inactive precursor proteins, or zymogens, contain small, amino terminus, homopolymeric regions of Asp that neutralize the cationic charge of the active protein during synthesis. After posttranslational cleavage, the anionic propeptide fragment may exhibit antimicrobial activity. To demonstrate this, ovine trypsinogen activation peptide, and frog (*Xenopus laevis*) PYL activation peptide, both containing homopolymeric regions of Asp, were synthesized and tested against previously described surfactant-associated anionic peptide. Peptides inhibited the growth of both gram-negative (MIC, 0.08 to 3.00 mM) and gram-positive (MIC, 0.94 to 2.67 mM) bacteria. Small, anionic, and charge-neutralizing propeptide fragments of zymogens form a new class of host-derived antimicrobial peptides important in innate defense.

Many defensins, hormones, and enzymes are synthesized as inactive precursor proteins, called zymogens. Zymogens consist of an amino terminus-inactivating prefix peptide (called a propeptide) followed by the active portion of the protein molecule (18). The propeptide fragment serves to prevent premature physiologic function of the active portion of the protein, thus protecting host cells from cytotoxic or enzymatic damage during enzyme synthesis and secretion (16, 18). For activation, zymogens are posttranslationally cleaved in a region amino terminal relative to the active site of the protein either before intracellular release of defensin into phagolysosomes (16) or before extracellular release of enzyme or hormone (18). While the synthesis, secretion, and activation mechanisms of these particular proteins have been well described (18), the fate and activity of the cleaved propeptide fragments are unknown in most instances (18). Some zymogen fragments have additional physiological functions in cascade reactions (e.g., peptides cleaved from complement and prothrombin [18, 27]) or in innate defense mechanisms (e.g., antimicrobial activity of gastric inhibitory polypeptide and diazepam-binding polypeptide [1]). The dual role of peptide fragments in innate host immunity has been proposed (3).

Some propeptide fragments of zymogens contain homopolymeric regions of Asp. These anionic regions in the propeptide fragment are conserved among a number of animal species (7, 18), as well as among a number of enzymes, hormones, and cationic proteins within an animal species. Recently, we isolated three small (721.6- to 823.8-Da), anionic peptides (called surfactant-associated anionic peptides or SAAP) from sheep pulmonary surfactant (6). These peptides inhibited the growth of both gram-negative and gram-positive bacteria in 0.14 M NaCl with 2.5 μM $ZnCl_2$. Antimicrobial activity resided in the core Asp hexapeptide homopolymeric region, and growth inhibition by a number of analogs increased with the number of Asp residues in the peptides (6). The amino acid sequences of SAAP are similar to those of charge-neutralizing activation peptides of Group I serine proteases (e.g., ovine trypsinogen activation peptide or TAP) (7, 18), some prohormones (e.g., frog [*Xenopus laevis*] PYL activation peptide [4, 12]), and prodefensins (16) and contain homopolymeric regions of Asp following a Gly or Ala terminating with Lys (Fig. 1). Preliminary work has shown that SAAP have a genetic basis of origin and may be propeptide fragments of a larger endogenous protein, heretofore unknown. Phage inserts, cloned from a degenerate nucleotide SAAP probe of a genomic sheep library, contained nucleotide sequences from which were derived predicted amino acid sequences of these homopolymeric Asp regions following Gly or Ala which appear to be only part of a much larger gene product. We propose that SAAP and similar small anionic propeptide fragments of zymogens (4, 10, 12, 16, 18) have innate immune functions in addition to their charge-neutralizing roles. While the antimicrobial activity of SAAP has been previously described (6), the antimicrobial activities of similar propeptide fragments have not. Therefore, the purpose of this study was to examine the antimicrobial activities of two representative synthetic propeptide fragments, ovine TAP and frog (*X. laevis*) PYL activation peptide, and to compare their activities with that of SAAP. Analogs of TAP and SAAP were also synthesized to assess the effect of peptide composition on antimicrobial activity.

SAAP (6), ovine TAP (7), and the propeptide fragment of hormone PYL from frog (*X. laevis*) skin (e.g., H-ADAD DDDDK-OH, starting 6 amino acids from the NH_2-terminal cleavage site for signal peptidase and immediately prior to the 24-amino-acid sequence for PYL [12]) were synthesized by Chiron Mimotopes Peptide Systems, San Diego, Calif., on a grafted polymer surface in a Multipin peptide synthesis format with *N*-alpha-Fmoc-protected amino acids. Side-chain deprotection and cleavage were carried out by acidolysis.

Peptides were purified on a Vydac (protein) C4 column (0.46 cm by 25 cm; 4-μm particle size; 300-Å core size). Fractions, in 0.1% (vol/vol) orthophosphoric acid in water, were eluted over 15 min with a 0 to 100% gradient of 0.1% (vol/vol) orthophosphoric acid in 60% (vol/vol) acetonitrile in water. Mass spectrometer analysis was performed with a Perkin-Elmer Sciex API III mass spectrometer having an ionspray ion source with ion-counting detection. Peptides were 95 to 99% pure. Analogs of TAP and SAAP (i.e., H-GDDDDDD-OH, H-ADDDDDD-OH, H-GADDDDD-OH, H-AADDDDD-

* Corresponding author. Mailing address: Respiratory and Neurologic Disease Research Unit, USDA, ARS, National Animal Disease Center, 2300 Dayton Rd., P.O. Box 70, Ames, IA 50010. Phone: (515) 239-8593. Fax: (515) 239-8458. E-mail: kbrogden@nadc.ars.usda.gov.

```
            Val Asp Asp Asp Asp Lys              TAP (sheep, goat, deer, cow)
        Ala Pro Asp Asp Asp Asp Lys              TAP (dogfish)
            Thr Asp Asp Asp Asp Lys              TAP (pig, horse)
Ala Asp Ala Asp Asp Asp Asp Asp Lys Arg Tyr      PYL
     Gly Asp Asp Asp Asp Asp Asp                 SAAP
     Asp Asp Asp Asp Asp Asp Asp                 SAAP
     Gly Ala Asp Asp Asp Asp Asp                 SAAP
         Thr Gln Asp Asp Gly Gly Lys             CAP
```

FIG. 1. Sequences of SAAP (6) and similar propeptide sequences found in other proenzymes and prohormones. TAP, released during the activation of trypsin (7, 18), is highly conserved throughout a number of animal species (7). H-ADADDDDDK-OH is part of the activation sequence for the prohormone precursor of PYL, a peptide isolated from frog (*X. laevis*) skin (12). Activation of prococoonase results in the proteolytic removal of a 1.7-kDa coccoonase activation peptide (CAP), which is thought to be cleaved further into two free Lys's, a tetrapeptide, and the heptapeptide shown (10).

OH, H-DDDDK-OH, and H-GDDDK-OH) were similarly prepared.

A panel of conventional gram-negative bacteria (*Klebsiella pneumoniae* ATCC 10031, *Escherichia coli* ATCC 12795, *Pseudomonas aeruginosa* ATCC 27312, and *Serratia marcescens* ATCC 14756) and gram-positive bacteria (*Staphylococcus aureus* ATCC 29213 and *Streptococcus faecalis* ATCC 29212) were used. *Pasteurella haemolytica* serotype A1 strain 82-25, isolated from a sheep with enzootic pneumonia, was also included since both SAAP and TAP are of sheep origin and nearly identical in composition (6, 7). A nonpathogenic eucaryotic organism, *Candida krusei* ATCC 6258, was also used. All cultures were grown in tryptose broth at 37°C for 3 h, pelleted by centrifugation at 5,900 × *g* for 15 min at 4°C, and resuspended in 0.14 M NaCl. The suspensions were adjusted in the spectrophotometer (78% transmittance; 600 nm; Coleman model 35; Bacharach Instrument Co.) to contain 1.0×10^8 CFU/ml and were diluted 10^5-fold to 10^3 CFU/ml with 0.14 M NaCl (6).

A dilution susceptibility test was used to obtain MICs (28). Since host-derived antimicrobial peptides and their analogs are sensitive to divalent cation concentration (14), salt concentration (3, 26), pH, and buffer composition (6, 9, 13), the following modifications were necessary to assess antimicrobial activity. Synthetic peptides were diluted twofold in 100 μl of 0.14 M NaCl on styrene plates (Immulon 1; Dynatech Laboratories, Inc., Chantilly, Va.). Then, 0.14 M NaCl with 10.0 μM $ZnCl_2$ (50 μl) was added since the bactericidal activity of SAAP is strongly dependent upon zinc as a cofactor (6). Finally, bacterial culture (50 μl) was added to each dilution, and the plates were incubated at 37°C for 120 min. Control wells containing only 0.14 M NaCl with 2.5 μM $ZnCl_2$ and bacteria were included. After incubation, the number of viable bacteria was determined by culturing 150 μl from each well in triplicate (50 μl/spot) on Trypticase soy agar containing 5% defibrinated sheep blood and by incubating the plates overnight at 37°C. MICs (millimolar values) were determined as the highest dilutions of peptides showing no growth on blood agar.

Since many antimicrobial peptides are inactivated by serum components (21–23), it was assumed that a carryover of peptide from the incubation mixture to the blood agar did not inhibit the growth of organisms surviving incubation in the test mixture, giving a false sense of killing. To demonstrate this, *P. haemolytica* was mixed with 0.14 M NaCl containing 2.5 μM $ZnCl_2$ or with 0.14 M NaCl containing 2.5 μM $ZnCl_2$ and 0.5 mM H-DDDDDDD-OH. Immediately after mixing (time 0), the number of viable bacteria was determined by culturing 150 μl from each well in triplicate (50 μl/spot) on Trypticase soy agar containing 5% defibrinated sheep blood and incubating the plates overnight at 37°C. *P. haemolytica* mixed with 0.14 M NaCl containing 2.5 μM $ZnCl_2$ had an average of 100.3 CFU/50-μl spot (standard error, 1.9 CFU/50-μl spot; $n = 30$ spots) and *P. haemolytica* mixed with 0.14 M NaCl containing 2.5 μM $ZnCl_2$ and 0.5 mM H-DDDDDDD-OH had an average of 101.0 CFU/50-μl spot (standard error, 1.5 CFU/50-μl spot; $n = 30$ spots).

TABLE 1. Antimicrobial activity of synthesized anionic peptide fragments

Organism	MIC[a] of synthetic peptides (mM)		
	TAP[b]	SAAP[c]	PYL[d]
P. haemolytica	0.16 (0.01)	0.08 (0.00)	0.23 (0.02)
E. coli	0.90 (0.16)	0.57 (0.11)	0.85 (0.12)
K. pneumoniae	0.85 (0.08)	0.80 (0.21)	0.90 (0.08)
P. aeruginosa	1.50	1.50	1.50
S. marcescens	3.00	1.50	1.50
S. aureus	1.08 (0.11)	0.94 (0.14)	1.80 (0.17)
S. faecalis	2.67 (0.17)	1.16 (0.13)	1.80 (0.17)
C. krusei	>2.77	>3.19	>5.11

[a] MICs followed by parenthetical values are means of six replications (standard error). MICs without parenthetical values are the results of only one replication.

[b] Ovine trypsinogen activation peptide synthesized from the sequence reported by de Haen et al. (7). Sequence, H-VDDDDK-OH; molecular mass, 705.7 Da.

[c] Respiratory-associated anionic peptide synthesized from the sequence previously reported (6). Sequence, H-DDDDDDD-OH; molecular mass, 823.6 Da.

[d] Propeptide fragment of the hormone PYL from frog (*X. laevis*) skin. Sequence, H-ADADDDDDK-OH, starting 6 amino acids from the NH_2-terminal cleavage site for signal peptidase and immediately prior to the 24-amino-acid sequence for PYL skin (12). Molecular mass, 978.5 Da.

To assess eucaryotic cytotoxicity, 100 μl of TAP, SAAP, or PYL (6.0 mM each) was added to 900 μl of B-lymphocyte leukemia cells (BL-3; ATCC CRL 8037; 1.1×10^6 cells/ml in McCoy's 5A medium with 10% fetal calf serum) and incubated for 18 h at 37°C with 5% CO_2. Propidium iodide (5 μg/ml) was added, and cell viability was assessed by flow-cytometric analysis (model XL cytometer; Coulter Corp.).

Activation peptides TAP and PYL inhibited the growth of bacteria, but neither peptide was cytocidal for BL-3 cells or *C. krusei* (Table 1). MICs varied between peptides and among bacterial species but were comparable to the MIC of SAAP. Gram-negative bacteria (e.g., *P. haemolytica*, *E. coli*, and *K. pneumoniae*) sensitive to surfactant-induced killing by normal serum (5) were more susceptible (MIC range, 0.08 to 0.90 mM peptide) than gram-positive bacteria (MIC range, 0.94 to 2.67 mM peptide). The MICs of peptide analogs for *P. haemolytica* serotype A1 strain 82-25 varied, depending upon the amino acid composition, from 30 to 200 μM (Table 2), similar to the variation previously reported with Asp dipeptide-to-heptapeptide homopolymers (6). Interestingly, gram-negative bacteria (e.g., *P. aeruginosa* and *S. marcescens*) resistant to surfactant-induced killing by normal serum (5) were slightly more resistant to all anionic peptides (MIC range, 1.50 to 3.00 mM peptide). The MICs of anionic peptides were comparable to those of other vertebrate antimicrobial peptides (3, 8, 11).

Innate extracellular immune mechanisms serve to suppress or reduce growth after microbial infection until nonspecific cellular and specific humoral and cellular immune mechanisms activate. In vivo, the efficacies of anionic peptides will depend upon the site of peptide release, peptide concentration, availability of zinc, comixture with serous fluid or plasma, rate of clearance by lymphatic, circulatory, and urinary systems, deg-

TABLE 2. MICs of synthesized SAAP and TAP analogs for *P. haemolytica* A1

Analog	MIC (mM)
TAP[a] analogs	
H-DDDDK-OH	0.09
H-GDDDK-OH	0.20
SAAP[b] analogs	
H-ADDDDDD-OH	0.05
H-GADDDDD-OH	0.03
H-AADDDDD-OH	0.03
H-GDDDDDD-OH	0.06

[a] Ovine trypsinogen activation peptide synthesized from the sequence reported by de Haen et al. (7).

[b] Surfactant-associated anionic peptide synthesized from the sequence previously reported (6).

radation by oligopeptidases, and type and concentration of microorganisms encountered. For example, the activation of pancreatic trypsinogen, which is produced by the pancreas acinar cells, is triggered by an enterokinase secreted from the brush border of the small intestine (18). TAP cleaved near the surface of the epithelial cells in the microenvironment of the mucous layer may act alone or in concert with other locally secreted defensins (e.g., Paneth cell defensins or cryptdins [20, 24]) to retard localized bacterial infection and invasion. PYL activation peptide in frog skin may simply add to the antimicrobial barrier already present in the skin and mucosal epithelium (17, 29).

In sites with limited circulatory drainage, such as the respiratory tract, SAAP may have more retention time in situ, may contribute to the anionic microenvironment on alveolar surfaces (2), and may be an adjunct to preexisting innate defense systems (25). Antimicrobial activity was found originally in pulmonary surfactant, but SAAP may also occur in the epithelial lining fluid or airway surface fluid removed from the lung by bronchoalveolar lavage. SAAP in the respiratory lining fluids would provide a barrier refractory to microbial infection and colonization.

The physiology of anionic peptides and charge-neutralizing propeptide fragments of zymogens has generally been ignored (18). These propeptide fragments are distinct from plant, insect, and vertebrate antimicrobial peptides (3, 15, 19), contain hydrophilic and acidic amino acids, are opposite in charge (pI 2.52 to 3.45), and are considerably smaller (<1 kDa) in size. In this report, we show that anionic propeptide fragments inhibit the growth of bacteria in vitro and we propose that they have innate immune functions on mucosal surfaces, in addition to their charge-neutralizing roles.

We thank Gwen Laird, Ginger Shipp, and Jonathan Seagren for technical assistance.

REFERENCES

1. **Agerberth, B., A. Boman, M. Andersson, H. Jornvall, V. Mutt, and H. Boman.** 1993. Isolation of three antibacterial peptides from pig intestines—gastric inhibitory polypeptide (7–42), diazepam-binding inhibitor (32–86) and a novel factor, peptide-3910. Eur. J. Biochem. **216:**623–629.
2. **Atwal, O. S., L. Viel, and K. J. Minhas.** 1990. An uptake of cationized ferritin by alveolar type I cells in airway-instilled goat lung: distribution of anionic sites on the epithelial surface. J. Submicros. Cytol. Pathol. **22:**425–432.
3. **Boman, H. G.** 1995. Peptide antibiotics and their role in innate immunity. Annu. Rev. Immunol. **13:**61–92.
4. **Brevins, C. L., and M. Zasloff.** 1990. Peptides from frog skin. Annu. Rev. Biochem. **59:**395–414.
5. **Brogden, K. A.** 1992. Ovine pulmonary surfactant induces killing of *Pasteurella haemolytica*, *Escherichia coli*, and *Klebsiella pneumoniae* by normal serum. Infect. Immun. **60:**5182–5189.
6. **Brogden, K. A., A. J. De Lucca, J. Bland, and S. Elliot.** 1996. Isolation of an ovine pulmonary surfactant-associated anionic peptide bactericidal for *Pasteurella haemolytica*. Proc. Natl. Acad. Sci. USA **93:**412–416.
7. **de Haen, C., H. Neurath, and D. C. Teller.** 1975. The phylogeny of trypsin-related serine proteases and their zymogens. New methods for the investigation of distant evolutionary relationships. J. Mol. Biol. **92:**225–259.
8. **Diamond, G., D. E. Jones, and C. L. Bevins.** 1993. Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene. Proc. Natl. Acad. Sci. USA **90:**4596–4600.
9. **Ellison, R. T., III, D. Boose, and F. M. LaForce.** 1985. Isolation of an antibacterial peptide from human lung lavage fluid. J. Infect. Dis. **151:**1123–1129.
10. **Felsted, R. L., K. J. Kramer, and J. H. Law.** 1973. Cocoonase. IV. Mechanism of activation of prococoonase from *Antheraea polyphemus*. J. Biol. Chem. **248:**3012–3020.
11. **Ganz, T., and R. I. Lehrer.** 1994. Defensins. Curr. Opin. Immunol. **6:**584–589.
12. **Hoffmann, W., K. Richter, and G. Kreil.** 1983. A novel peptide designated PYLa and its precursor as predicted from cloned mRNA of *Xenopus laevis* skin. EMBO J. **2:**711–714.
13. **LaForce, F. M., and D. S. Boose.** 1984. Effect of zinc and phosphate on an antibacterial peptide isolated from lung lavage. Infect. Immun. **45:**692–696.
14. **Lehrer, R. I., T. Ganz, D. Szklarek, and M. E. Selsted.** 1988. Modulation of the in vitro candidacidal activity of human neutrophil defensins by target cell metabolism and divalent cations. J. Clin. Invest. **81:**1829–1835.
15. **Martin, E., T. Ganz, and R. I. Lehrer.** 1995. Defensins and other endogenous peptide antibiotics of vertebrates. J. Leukocyte Biol. **58:**128–136.
16. **Michaelson, D., J. Rayner, M. Couto, and T. Ganz.** 1992. Cationic defensins arise from charge-neutralized propeptides: a mechanism for avoiding leukocyte autocytotoxicity? J. Leukocyte Biol. **51:**634–639.
17. **Moore, K. S., C. L. Bevins, N. Tomassini, K. M. Huttner, K. Sadler, J. E. Moreira, J. Reynolds, and M. Zasloff.** 1992. A novel peptide-producing cell in *Xenopus*: multinucleated gastric mucosal cell strikingly similar to the granular gland of the skin. J. Histochem. Cytochem. **40:**367–378.
18. **Neurath, H., and K. A. Walsh.** 1976. Role of proteolytic enzymes in biological regulation (a review). Proc. Natl. Acad. Sci. USA **73:**3825–3832.
19. **Nicolas, P., and A. Mor.** 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. **49:**277–304.
20. **Ouellette, A. J., and M. E. Selsted.** 1996. Paneth cell defensins: endogenous peptide components of intestinal host defense. Fed. Am. Soc. Exp. Biol. J. **10:**1280–1289.
21. **Panyutich, A., and T. Ganz.** 1991. Rapid Communication-Activated alpha2-macroglobulin is a principal defensin-binding protein. Am. J. Respir. Cell Mol. Biol. **5:**101–106.
22. **Panyutich, A. V., P. S. Hiemstra, S. van Wetering, and T. Ganz.** 1995. Human neutrophil defensin and serpins form complexes and inactivate each other. Am. J. Respir. Cell Mol. Biol. **12:**351–357.
23. **Panyutich, A. V., O. Szold, P. H. Poon, Y. Tseng, and T. Ganz.** 1994. Identification of defensin binding to C1 complement. FEBS Lett. **356:**169–173.
24. **Selsted, M. E., S. I. Miller, A. H. Henschen, and A. J. Ouellette.** 1992. Enteric defensins: antibiotic peptide components of intestinal host defense. J. Cell Biol. **118:**929–936.
25. **Sherman, M. P., and T. Ganz.** 1992. Host defense in pulmonary alveoli. Annu. Rev. Physiol. **54:**331–350.
26. **Smith, J. J., S. M. Travis, E. P. Greenberg, and M. J. Welsh.** 1996. Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85:**229–236.
27. **Takabayashi, T., E. Vannier, B. D. Clark, H. H. Margolis, C. A. Dinarello, J. F. Burke, and J. A. Gelfand.** 1996. A new biologic role of C3a and C3aArg-regulation of TNF-α and IL-β synthesis. J. Immunol. **156:**3455–3460.
28. **Woods, G. L., and J. A. Washington.** 1995. Antibacterial susceptibility tests: dilution and disk diffusion methods, p. 1327–1341. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology. ASM Press, Washington, D.C.
29. **Zasloff, M.** 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:**5449–5453.