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Selective Inhibition of Microbial Serine Proteases by eNAP-2, an Antimicrobial Peptide from Equine Neutrophils

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Equine neutrophil antimicrobial peptide 2 (eNAP-2), a recently described antimicrobial peptide isolated from equine neutrophils, was found to selectively inactivate microbial serine proteases (subtilisin A and proteinase K) without inhibiting mammalian serine proteases (human neutrophil elastase, human cathepsin G, and bovine pancreatic trypsin). Although the primary structure of eNAP-2 resembled that of several known antiproteases that belong to the 4-disulfide core peptide family, this pattern of selectivity is unique. eNAP-2 formed a noncovalent complex with native subtilisin A or proteinase K but did not associate with these enzymes if they had been treated with phenylmethylsulfonyl fluoride, a serine protease inhibitor. The eNAP-2–microbial protease complex was disrupted by boiling or by exposure to low pH. We suggest that eNAP-2 exerted selective antiproteinase activity by binding tightly but noncovalently to the active site of subtilisin A or proteinase K. Since microbial exoproteases may act as virulence factors, the combined antimicrobial and antiprotease activities of eNAP-2 could allow it to play an important role in neutrophil-mediated antimicrobial defenses.

Neutrophils, the most abundant phagocytes in blood, contain a variety of antimicrobial peptides and proteins that equip them to kill ingested microorganisms. We recently purified a novel peptide, equine neutrophil antimicrobial peptide 2 (eNAP-2), from acid-extracted equine neutrophil granules and described both its antibiotic properties and N-terminal sequence (1). Its structural homology to the 4-disulfide core family, some of whose members are antiproteases, led us to test the ability of eNAP-2 to inhibit serine proteases. We found that eNAP-2 was a potent and selective inhibitor of the microbial serine proteases subtilisin A and proteinase K.

MATERIALS AND METHODS

eNAP-2. eNAP-2 was purified from equine blood or from sterile uterine exudates as described previously (1, 2). Briefly, peripheral blood neutrophils (polymorphonuclear leukocytes) were isolated on a discontinuous Percoll density gradient and disrupted by nitrogen cavitation; disruption was followed by low-speed centrifugation, which yielded a granule-rich postnuclear supernatant. The cytoplasmic granules were recovered by centrifugation at 27,000 × *g* for 20 min at 2°C, extracted in ice-cold 10% acetic acid, and concentrated by vacuum centrifugation. eNAP-2 that had been purified to apparent homogeneity by gel permeation and reversed-phase high performance liquid chromatography (2) was lyophilized and redissolved in 0.01% acetic acid for use in these studies. Human and rabbit defensin peptides used as controls in these experiments had been previously isolated and purified in our laboratory (4, 16). Hen egg white lysozyme was purchased from Sigma. Peptide concentrations were estimated spectrophotometrically by applying the formula $(A_{215} - A_{225}) \times 144$ = protein concentration (micrograms per milliliter) (18).

Proteases. Subtilisin A (EC 3.4.21.14; M_r, 27,300) prepared from *Bacillus licheniformis* was purchased from both Calbiochem, La Jolla, Calif., and Sigma Chemicals, St. Louis, Mo.; proteinase K (EC 3.4.21.14; M_r, 18,500), purified from the fungus *Tritirachium album*, was obtained from both U.S. Biochemical Corp., Cleveland, Ohio, and Boehringer-Mannheim, Indianapolis, Ind. Elastase (EC 3.4.21.37; M_r, 29,500) and cathepsin G (EC 3.4.21.20; M_r, 23,500), purified from human neutrophils, were purchased from Art Biochemicals, Athens, Ga., and bovine pancreatic trypsin (EC 3.4.21.4; M_r, 23,300) was obtained from Boehringer-Mannheim.

Substrates. The synthetic tetrapeptides *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (AAPFpna) and *N*-methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine *p*-nitroanilide (AAPVpna) and sulfanilamide-azocasein substrates were purchased from Sigma.

Enzyme assays. Amidase activity was measured with the synthetic tetrapeptide substrates and reaction buffers shown in Table 1. Inhibition was tested by adding increasing amounts of eNAP-2 ranging from 1.25 ng to 1.0 μg (dissolved in 0.01% acetic acid) to plastic cuvettes that contained 20 μl of serine protease (1 μg/ml) in reaction buffer. Such preincubations were carried out for 30 s to 30 min at 0, 25, or 37°C and at pHs ranging from 5.5 to 11.5. Various controls for eNAP-2 were studied in the same manner, including 0.01% acetic acid, human defensin HNP-1, rabbit defensin NP-2, and hen egg white lysozyme. The enzymatic reactions were initiated by adding 570 μl of 1 mM substrate (Table 1), and the progress of the reaction was monitored by measuring the optical density at 410 nm. Changes in the optical density at 410 nm were recorded at 1-min intervals for 30 min at 24.5°C with a DU-8 spectrophotometer (Beckman) equipped with a temperature-controlled cell holder and a Kinetics II module.

Azocasein was used to measure the ability of eNAP-2 to inhibit proteolysis by subtilisin A and proteinase K. In such experiments, 6 μg of enzyme was combined with 1.25 μg of lyophilized eNAP-2 (approximately an equimolar ratio) or with 1 μg of rabbit defensin NP-1 (control) in 60 μl of reaction buffer. The mixtures were preincubated for 15 min at 0 or 25°C. Subsequently, 1 ml of 2% sulfanilamide–

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TABLE 1. Serine proteases and substrates

Protease	Substrate	Reaction buffer
Microbial		
Subtilisin	1 mM AAPFpna	0.08 M sodium phosphate (pH 7.8) or 10 mM sodium phosphate (pH 7.4)
	2% azocasein	0.1 M Tris–0.5 M NaCl (pH 7.5)
Proteinase K	1 mM AAPFpna	0.08 M sodium phosphate (pH 7.8) or 10 mM sodium phosphate (pH 7.4)
	2% azocasein	0.1 M Tris–0.5 M NaCl (pH 7.5)
Mammalian		
Human PMN[a] elastase	1 mM AAPVpna	0.1 M Tris–0.5 M NaCl (pH 7.5)
Human PMN cathepsin G	1 mM AAPFpna	0.1 M Tris (pH 8.3)
Bovine pancreatic trypsin	1 mM AAPFpna	0.1 M Tris (pH 8.3)

[a] PMN, polymorphonuclear leukocyte.

azocasein substrate in reaction buffer (Table 1) was added, and the mixture was incubated further at 25°C. Aliquots were removed at 1, 5, and 24 h, placed into microcentrifuge tubes, and assessed for endopeptidase activity by using a modification of a previously described technique (14). Briefly, after the enzyme, enzyme-inhibitor complex, or enzyme-control mixture had been incubated with the substrate, aliquots of the mixture were removed, mixed with 10% trichloroacetic acid (Sigma), and centrifuged at 8,000 × *g* for 3 min, after which the precipitate and supernatant were separated. NaOH (final concentration, 0.5 N) was added to the supernatant to enhance color development, and the resulting solution was assessed for A_{440}.

Enzyme-inhibitor complex formation. We used several polyacrylamide gel electrophoresis (PAGE) systems (Table 2) to characterize the formation of a complex between the microbial proteases and eNAP-2. Denaturing and nondenaturing conditions were used for acidic gels (9, 13) and for sodium dodecyl sulfate (SDS)-Tricine-PAGE (15); reducing and nonreducing conditions were used for SDS-Tricine-PAGE. SDS-Tricine-PAGE minigels were run at 33 mA for approximately 2 h on an electrophoresis unit (model SE-250; Hoefer Scientific Instruments, San Francisco, Calif.). The approximate stoichiometry of the eNAP-2–enzyme complex was determined by incubating a constant amount of eNAP-2 with increasing amounts of subtilisin A and proteinase K, up to a calculated 2:1 (enzyme/inhibitor) molar ratio. In some experiments, identical amounts of these enzymes had been exposed to 1 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, prior to incubation with eNAP-2.

RESULTS

Antiprotease selectivity. Preincubation of eNAP-2 with the microbial proteinase subtilisin A or proteinase K abolished both the amidase and endopeptidase activities of these enzymes (Fig. 1). In contrast, preincubation of a fourfold molar excess of eNAP-2 with several serine proteases of mammalian origin, including human neutrophil elastase, human cathepsin G, and bovine pancreatic trypsin, did not inhibit their enzymatic activity (data not shown). Preincubation of eNAP-2 with microbial proteases for 1 or 3 min resulted in suboptimal enzyme inhibition, whereas preincubation for 8 min eliminated enzymatic activity (Fig. 2). When, instead of being preincubated with the microbial protease, eNAP-2 was added 7 min after the enzyme and substrate were mixed, the inhibitory effects of eNAP-2 developed over several minutes, consistent with a slow formation of an enzyme-inhibitor complex (Fig. 3).

Enzyme-inhibitor complex formation. We demonstrated that eNAP-2 formed a complex with subtilisin A or proteinase K by performing SDS-Tricine-PAGE without boiling the samples (Fig. 4). When, however, such samples were boiled, the complex dissociated (data not shown). Development of a stable complex was apparent when PAGE was performed at pH 4.3 or 5.7 in the absence of SDS but not when PAGE was performed at pH 2.5 (data not shown). Although complex formation was complete at a calculated enzyme/inhibitor molar ratio of 2:1, the substantial autodegradation of the proteases (see below) suggested that the actual stoichiometry of the complex between intact enzyme and eNAP-2 was

TABLE 2. PAGE analysis of eNAP-2 and microbial serine proteases

Gel system[a]	pH	Denaturing agent	Reducing agent	Enzyme-inhibitor complex
Acidic gels				
Acrylamide, HOAc[b]	2.5	None	None	Dissociated
Acrylamide, HOAc, 1.0 or 5.0 M urea	2.5	Urea	None	Dissociated
Acrylamide, HOAc, KOH	4.3	None	None	Intact
Acrylamide, HOAc, KOH	5.7	None	None	Intact
SDS-Tricine gels				
Acrylamide, glycerol, Tris, SDS	8.25	Heat	DTT[c]	Dissociated
Acrylamide, glycerol, Tris, SDS	8.25	None	None	Intact
Acrylamide, glycerol, Tris, SDS	8.25	Heat	None	Dissociated

[a] Dimensions of all minigels, 8.0 by 7.0 by 0.75 cm.
[b] HOAc, acetic acid.
[c] DTT, dithiothreitol.

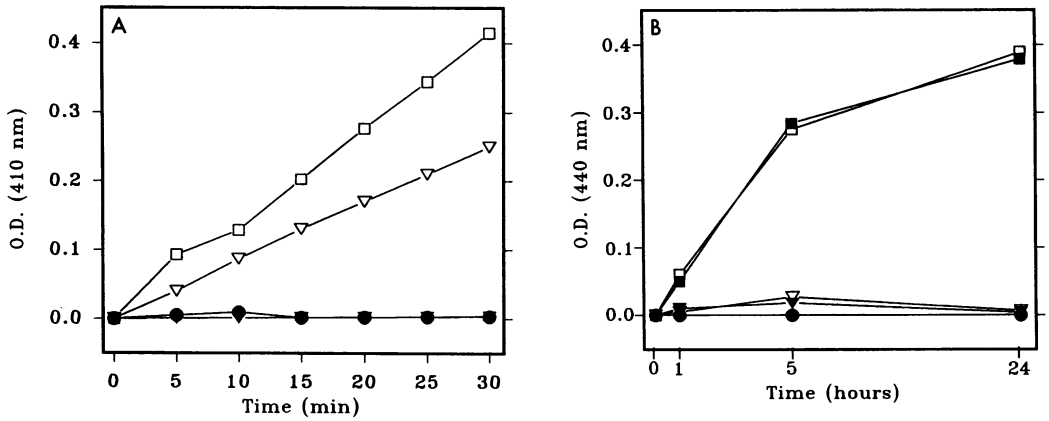

FIG. 1. (A) Inhibition of the amidase activity of subtilisin A and proteinase K by eNAP-2 (AAPFpna substrate). A molar excess of eNAP-2 (35 ng) was preincubated for 15 min with 5 ng each of the microbial proteases subtilisin A (▼) and proteinase K (●) in 35 μl of 10 mM sodium phosphate buffer (pH 7.4) before substrate was added. Control samples containing 5 ng of subtilisin A (□) or proteinase K (▽) were also tested. O.D. (410 nm), optical density at 410 nm. (B) Inhibition of the endopeptidase activity of subtilisin A by eNAP-2 (azocasein substrate). eNAP-2 (1.25 μg) was preincubated for 15 min with 6 μg of subtilisin A at 0°C (▼) or 25°C (▽) in 60 μl of 0.1 M Tris (pH 7.5) before substrate was added. The controls included an enzyme-free blank (●), inhibitor-free subtilisin (□), and subtilisin (6 μg) preincubated for 15 min at 0°C with 1 μg of rabbit defensin NP-1 (■). Experiments were performed in triplicate. Panels A and B depict data from a single representative experiment. O.D. (440 nm), optical density at 440 nm.

more probably 1:1. When analyzed by SDS-Tricine-PAGE without prior treatment with PMSF, both subtilisin A and proteinase K underwent marked spontaneous autolysis (Fig. 4, lanes 5 and 11). It has been shown that SDS can increase the activity of proteinase K as much as sevenfold over the activity exhibited in the absence of SDS (8). No significant protease autodegradation was observed after subtilisin A and proteinase K bound to eNAP-2, and, as expected, preincubation with PMSF also protected both proteases from autodegradation (lanes 6 and 12). Pretreatment of the proteases with PMSF completely prevented the subsequent formation of a complex with eNAP-2 (data not shown). All of these observations suggest that the active-site serine participated in the eNAP-2–protease interaction.

DISCUSSION

The presence in equine neutrophils of cationic peptides that selectively inhibited the activity of subtilisin A and proteinase K was previously reported by Pellegrini et al. (10). Although no information was provided about the amino acid sequence or antimicrobial activity of these peptides, their M_r was reported to range between 6,300 and 7,400. eNAP-2, a cationic peptide whose M_r is approximately 6,500, fits this general description perfectly (1).

Two constellations of serine proteases have been described: the subtilisin and chymotrypsin superfamilies (7). Whereas the subtilisin superfamily, whose members include

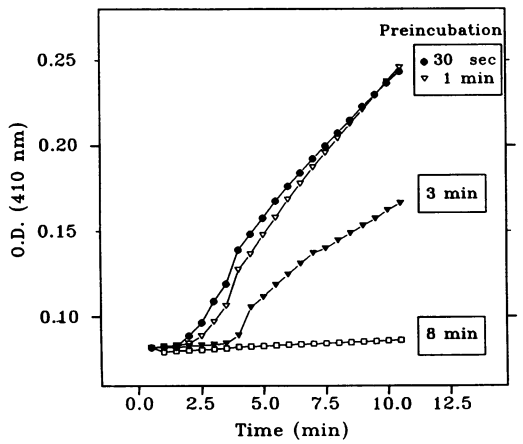

FIG. 2. Effect of preincubation time on inhibition. Approximately equimolar amounts of eNAP-2 (6.25 ng) and subtilisin (20 ng) were incubated together on ice in 30 μl of 0.1 M Tris (pH 7.5) for 30 s or 1, 3, or 8 min. Thereafter, 570 μl of 1 mM AAPFpna substrate at 37°C was added, and its hydrolysis was monitored by measuring its optical density at 410 nm [O.D. (410 nm)]. Preincubation for 8 min resulted in complete inactivation.

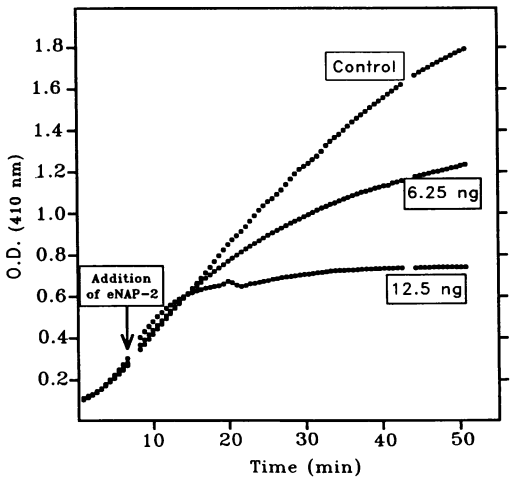

FIG. 3. Kinetics of inhibition. After the hydrolysis of AAPFpna by subtilisin (10 ng) was monitored for approximately 7 min at 37°C, eNAP-2 was added. The final assay volume was 600 μl. Whereas addition of 12.5 ng of eNAP-2 markedly inhibited enzymatic activity within 10 min, addition of 6.25 ng of eNAP-2 caused a more gradual and less pronounced effect. O.D. (410 nm), optical density at 410 nm.

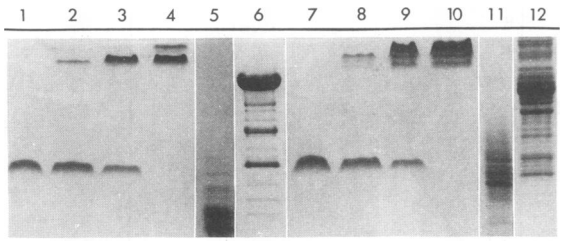

FIG. 4. Formation of an enzyme-inhibitor complex. A constant amount of eNAP-2 (1.25 μg) was preincubated with increasing amounts of subtilisin or proteinase K for 30 min at 0°C in 10 μl of 0.1 M Tris buffer (pH 7.5). Lanes: 1, eNAP-2; 2 to 4, eNAP-2 that had been preincubated with 1, 5, or 10 μg, respectively, of subtilisin; 5, subtilisin (20 μg); 6, PMSF-treated subtilisin (20 μg); 7, eNAP-2; 8 to 10, eNAP-2 that had been preincubated with 1, 5, or 10 μg, respectively, of proteinase K; 11, proteinase K (20 μg); 12, PMSF-treated proteinase K (20 μg). Note that both subtilisin and proteinase K exhibited considerable autodegradation in the absence of prior treatment with PMSF (lanes 5 and 11). In this composite illustration, lanes 1 to 4 and 7 to 10, lanes 5 and 11, and lanes 6 and 12 are derived from three separate nondenaturing and nonreducing SDS–Tricine–16.5% polyacrylamide gels stained with Coomassie brilliant blue.

subtilisin A and proteinase K, is expressed only in bacteria and fungi, the chymotrypsin superfamily is broadly represented among microorganisms, plants, and animals. Although both superfamilies contain serine, histidine, and aspartic acid residues in their active sites, their three-dimensional structures differ substantially (7). These differences probably underlie the selective inhibition of subtilisin-related proteases by eNAP-2.

Addition of eNAP-2 to the microbial proteases not only abolished their amidase and endopeptidase activities but also led to the formation of an approximately equimolar eNAP-2–enzyme complex that was stable over a broad range of pH (from 5.5 to 11.5) and temperature (from 0 to 37°C). The complex was dissociated by boiling or by exposure to pH 2.5, indicating its noncovalent nature. The protein and peptide inhibitors of serine proteases were grouped into 10 families by Laskowski and Kato (6). Two of these, the pancreatic trypsin inhibitor, or Kunitz family, and the pancreatic secretory trypsin inhibitor, or Kazal family, have been especially well studied. Both of these consist of relatively small, cysteine-rich peptides that are composed of about 60 amino acid residues and contain multiple intramolecular disulfide bonds. These inhibitors bind tightly to the active site of their cognate enzyme, but their subsequent dissociation occurs slowly, if at all (11). Although eNAP-2 is not a Kunitz or Kazal family member, these general features are quite consistent with its effects on microbial serine proteases.

Exoproteases reportedly act as virulence factors for a variety of microorganisms including *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Candida albicans* (3, 5, 12). Not only may some microbial proteases damage host tissues directly, but also others may cause damage indirectly by activating endogenous interstitial procollagenases in fibroblasts and neutrophils (17).

Since eNAP-2 and the microbial proteases formed a stable complex over a wide range of conditions of pH, osmolarity, and temperature, they are also likely to do so in vivo. Because eNAP-2 exists at a moderately high concentration within the cytoplasmic granules of equine neutrophils (1), its secretion into phagolysosomes or its extracellular release could defend these compartments from the effects of unopposed microbial proteases. The dual antimicrobial and antiprotease activities of eNAP-2 may allow it to play an important role in equine host defense against infection.

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