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A peptide derived from human bactericidal/permeability-increasing protein (BPI) exerts bactericidal activity against Gram-negative bacterial isolates obtained from clinical cases of bovine mastitis☆

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Abstract

Gram-negative bacteria are responsible for approximately one-third of the clinical cases of bovine mastitis and can elicit a life-threatening, systemic inflammatory response. Lipopolysaccharide (LPS) is a membrane component of Gram-negative bacteria and is largely responsible for evoking the inflammatory response. Antibiotic and anti-inflammatory therapy for treating Gram-negative infections remains suboptimal. Bactericidal/permeability-increasing protein (BPI) is a neutrophil-derived protein with antimicrobial and LPS-neutralizing properties. Select peptide derivatives of BPI are reported to retain these properties. The objective of this study was to evaluate the antimicrobial activity of a human BPI-derived synthetic peptide against clinical bovine mastitis isolates of Gram-negative bacteria. A hybrid peptide was synthesized corresponding to two regions of human BPI (amino acids 90–99 and 148–161), the former of which has bactericidal activity and the latter of which has LPS-neutralizing activity. The minimum inhibitory (MIC) and bactericidal (MBC) concentrations of this peptide against various genera of bacteria were determined using a broth microdilution assay. The MIC's were determined to be: 16–64 μg/ml against *Escherichia coli*; 32–128 μg/ml against *Klebsiella pneumoniae* and *Enterobacter* spp.; and 64–256 μg/ml against *Pseudomonas aeruginosa*. The MBC's were equivalent to or 1-fold greater than corresponding MIC's. The peptide had no growth inhibitory effect on *Serratia marcescens*. The antimicrobial activity of the peptide was retained in the presence of serum, but severely

Abbreviations: ATCC, American Type Culture Collection; BPI, bactericidal/permeability-increasing protein; BPI_{pep}, BPI-derived peptide; CBS, citrate-buffered saline; $Ctrl_{pep}$, control peptide; EDTA, ethylenediaminetetraacetic acid; LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MW, molecular weight

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impaired in milk. Further functional evaluation of the peptide demonstrated its ability to completely neutralize LPS. Together, these data support additional investigations into the therapeutic application of BPI to the treatment of Gram-negative infections in cattle.
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1. Introduction

Approximately 20–30% of all clinical cases of bovine mastitis are caused by Gram-negative bacteria, and it is estimated that 10–30% of these clinical cases develop into life-threatening, peracute mastitis (Erskine et al., 1991; Ziv, 1992). The deleterious outcome associated with severe Gram-negative intramammary infections is due, in part, to the systemic inflammatory response elicited by lipopolysaccharide (LPS), a component of the outer bacterial membrane. Current antibiotic therapy for the treatment of these infections remains suboptimal, as do therapeutic options for counteracting the excessive inflammatory response elicited by LPS.

Bactericidal/permeability-increasing protein (BPI) is a neutrophil-derived, cationic protein that possesses bactericidal and LPS-binding activity (Elsbach, 1998). There is ~63% amino acid homology between the human, rabbit, and bovine orthologs (Leong and Camerato, 1990; Beamer et al., 1998), however, little is known about the activity of bovine BPI. Recombinant human BPI has been shown to have therapeutic efficacy for the treatment of Gram-negative infections (Kelly et al., 1993; Rogy et al., 1994; Levin et al., 2000). The amino-terminal half of human BPI (Gazzano-Santoro et al., 1992), as well as synthetic peptides corresponding to amino acid sequences 65–99 and 142–169, retain antibacterial and LPS-neutralizing properties (Gray and Haseman, 1994; Little et al., 1994; Little, 2002; Jiang et al., 2004).

The objective of the current study was to evaluate a peptide with a sequence corresponding to two regions within human BPI for its growth inhibitory activity towards various bacteria that induce clinical bovine mastitis. To our knowledge, this is the first study to assess the activity of this peptide against clinical mastitis bacterial isolates and to evaluate its function in the physiologically relevant fluids of bovine sera and milk.

2. Materials and methods

2.1. BPI-derived peptide (BPI_{pep})

A peptide [(KWKAQKRFLKKSKVGWLIQLFHKK) (MW: 3027 g/mol)] corresponding to two discontinuous regions of sequence within the mature form of human BPI [amino acids 90–99 (underlined) and 148–161] was commercially synthesized (Genemed Synthesis, Inc., South San Francisco, CA). Control peptides [(MCHWAGGASNTGDARGDVFGKQAG) (MW: 2394 g/mol)]; [(KAKAQKRFLKKSKVGALIQAFHKK) (MW: 2755 g/mol)]; and [(KSKVGWLIQLFHKKKWKAQKRFLK) (MW: 3027 g/mol)] were synthesized by the same company. High-performance liquid chromatography (HPLC) and mass spectroscopy (MS) analysis were performed by the commercial supplier to evaluate peptide purity and confirm sequence identity (data not shown). The purities of the peptides ranged between 95 and 98%. The peptides were reconstituted in citrate-buffered saline [CBS; 20 mM sodium citrate, 150 mM sodium chloride, 0.1% pluronic F-68, and 0.002% polysorbate 80, pH 5.0 (Sigma Chemical Co., St. Louis, MO)] up to a final maximal concentration of 10 mg/ml, aliquotted, and stored at −20 °C. CBS has been used by others to reconstitute BPI and its derivatives (Lin et al., 1996; Abrahamson et al., 1997; Beamer, 2002), and the inclusion of pluronic F-68 and polysorbate 80 surfactants in CBS has been reported to improve peptide stability and enhance resistance to aggregation, particle formation and precipitation (McGregor et al., 1996). For those peptides that contained aromatic amino acids, solubility in CBS was confirmed by measuring the absorbance of the dissolved peptides at 280 nm on a Beckman DU® 530 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). All aliquots of the reconstituted peptides were thawed only once and diluted immediately prior to use.

2.2. Bacterial strains

Isolates of *Escherichia coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, and *Enterobacter aerogenes*, were used to assess the bactericidal activity of peptides in a broth microdilution assay. All isolates were obtained from clinical cases of bovine mastitis and held in repositories at Cornell University (gift of Dr. Y.H. Schukken; Quality Milk Production Services Program, Cornell University, Ithaca, NY) or the USDA Beltsville Agricultural Research Center. *E. coli* strain P4 (gift of Dr. A.J. Bramley; Institute for Animal Health, Compton Laboratory, Newbury, England), which was also originally isolated from a clinical case of mastitis (Bramley, 1976), was used to assess the bactericidal activity of BPI_{pep} in both the broth microdilution assay and in biological fluids. Reference strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213) were obtained from a commercial source [American Type Culture Collection (ATCC), Manassas, VA]. To obtain fresh inoculums for evaluation, a sterile loop of each bacterial glycerol stock was streaked on a blood agar plate. After overnight incubation at 35 °C, a single uniform colony was picked, streaked on a new blood agar plate, and incubated overnight at 35 °C.

2.3. Broth microdilution assay for antimicrobial susceptibility

To evaluate the antimicrobial activity of BPI_{pep}, the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of the peptide were determined using a standardized broth microdilution assay. In addition to the mastitis isolates, *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213) reference strains were evaluated. The MIC and MBC values of tetracycline (MW: 480.90 g/mol) (Sigma Chemical Co.) for the various bacteria were determined as well. The assays were performed in accordance with Clinical and Laboratory Standards Institute (CLSI) [formerly National Committee for Clinical Laboratory Standards (NCCLS)] guidelines (NCCLS, 1999, 2002). First, a single colony of each fresh bacterial inoculum was diluted in cation-adjusted Mueller-Hinton broth (BD Biosciences, Franklin Lakes, NJ) and the suspension adjusted to achieve a transmittance equivalent to a 0.5 McFarland standard (10^8 CFU/ml) using a colorimeter. The bacterial suspension was then diluted 100-fold in cation-adjusted Mueller-Hinton broth to reach a final inoculum concentration of 10^6 CFU/ml. Fifty microliters of the inoculum were added to individual wells within a 96-well round bottom plate containing 50 μl of either broth alone, or broth containing serially diluted peptide or tetracycline. A negative control column was included in the plate that included 100 μl of broth alone. The plates were incubated for 18 h at 35 °C with ambient air circulation and without shaking. The wells were subsequently inspected for cloudiness and pellet formation using a magnifying glass plate reader (Dynatech Laboratories, Inc., Alexandria, VA). The lowest concentration at which no pellet or cloudiness was apparent in a given well was identified as the MIC. The MBC values were determined by plating 10 μl aliquots from the 96-well plates used to determine MIC values onto blood agar plates. The plates were incubated overnight at 35 °C and colonies subsequently enumerated. The MBC values were determined as the lowest concentration at which ≥99.9% of inoculated bacteria were killed.

2.4. Serum and whey preparation

Milk and blood samples were aseptically collected from eight clinically healthy, primiparous Holstein cows in mid-lactation with milk somatic cell counts of <150,000 cells/ml. All cows were determined to be free of intramammary pathogens by the absence of bacterial growth on blood agar plates spread with milk samples aseptically collected from each animal on 3 different days. For the preparation of whey, milk samples were centrifuged at 44,000 × *g* at 4 °C for 30 min, and the fat layer removed with a spatula. The skim milk was transferred into another sterile tube, centrifuged for an additional 30 min, and the translucent supernatant collected. Milk and whey samples were pasteurized by heating at 63 °C for 30 min. For the preparation of serum, blood was collected from the coccygeal vein into vacutainer tubes containing gel and clot activator (BD Biosciences). The tubes were inverted ×5, the blood allowed to clot for 30 min, and the tubes centrifuged at 1500 × *g* for 15 min. The clear serum supernatants were aseptically transferred into sterile tubes and

heat-inactivated by incubation at 56 °C for 30 min. An aliquot of each sample was plated on blood agar plates and samples that were free of detectable bacterial growth were stored at −20 °C.

2.5. Assay of antimicrobial activity in serum, milk, or whey

Overnight bacterial cultures of *E. coli* strain P4 grown in trypticase soy broth were diluted 1:1000 in brain heart infusion broth and incubated at 37 °C for 2 h while shaking at 225 rpm. The log-phase bacteria were diluted 1:10 in brain heart infusion broth to obtain a final concentration of 1×10^6 CFU/ml. The bacterial inoculum (0.01 ml) was added to a 1.5 ml sterile microcentrifuge tube containing 0.04 ml of serum, milk, or whey. A 0.05 ml aliquot of either peptide or CBS alone was added to each tube. The samples were then shaken at 100 rpm for 6 h at 37 °C after which they were serially diluted in sterile phosphate-buffered saline and plated on MacConkey agar plates (BD Biosciences). The plates were incubated overnight at 37 °C and the colonies enumerated. For assays examining the effect of ethylenediaminetetraacetic acid (EDTA; MW: 372.24 g/mol) (Quality Biological, Inc., Gaithersburg, MD) or cations on BPI_{pep} antimicrobial activity in milk or whey, the same procedure was followed as above except that 0.025 ml aliquots of varying concentrations EDTA or cations [$CaCl_2$ (MW: 110.98 g/mol) or $MgCl_2$ (MW: 95.21 g/mol); Sigma Chemical Co.] were added to milk or whey, respectively, followed by the addition of 0.01 ml inoculums of bacteria and 0.025 ml aliquots of either BPI_{pep} or CBS.

2.6. Assay for neutralization of LPS

The ability of BPI_{pep} or control peptides to neutralize LPS was determined using a commercially available *Limulus* amebocyte lysate (LAL) assay (Cambrex BioScience Walkersville, Inc., Walkersville, MD). Polymyxin B (MW: 1385.61 g/mol) (Sigma Chemical Co.), which has been demonstrated to bind and neutralize LPS (Cooperstock, 1974; Bannerman et al., 1998), was evaluated in parallel. Increasing concentrations of peptides or polymyxin B were incubated with 1 ng of highly purified LPS, derived from *E. coli* 0111:B4 (Sigma Chemical Co.), in a 500 μl reaction volume of endotoxin-free water for 30 min at 37 °C while shaking at 100 rpm. Following incubation, the amount of free LPS was determined in each sample according to the manufacturer's instructions. Briefly, 50 μl of the above mixture were placed into individual wells of a 96-well microtiter plate and the reaction initiated by the addition of an equal volume of amebocyte lysate. Following incubation at 37 °C for 10 min, 100 μl of chromogenic substrate was added and the plate incubated for 5–10 min at 37 °C. The reaction was stopped by the addition of 50 μl of glacial acetic acid and the absorbance measured at a wavelength of 405 nm on a microplate reader (Biotec Instruments, Inc., Winooski, VT). The amount of non-bound LPS was extrapolated from a standard curve and the percent inhibition calculated according to the following formula:

$$\frac{[(\text{amount of free LPS in control samples}) - (\text{amount of free LPS in test samples})] \times 100}{\text{amount of free LPS in control samples}}$$

2.7. Statistical methods

For studies investigating the effect of EDTA or cations on BPI antimicrobial activity, a one-way analysis of variance was used to compare the mean responses between experimental groups. The Dunnett *post hoc* comparison test was used to compare values among experimental conditions to designated control groups. Statistical analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, Inc., San Diego, CA). A P-value of <0.05 was considered significant.

3. Results and discussion

3.1. BPI_{pep} synthesis

Peptide mapping has identified regions within amino acids 65–99 and 142–169 of human BPI as conferring bactericidal and LPS-neutralizing activity, respectively (Little et al., 1994). Within amino acids 65–99, bactericidal activity has been shown to be

conferred by a subset region within amino acids 85–99 and a synthetic peptide corresponding to amino acids 90–99 of human BPI has been demonstrated to possess bactericidal activity (Gray and Haseman, 1994; Little et al., 1994). Several regions within BPI confer LPS-binding capability and a peptide corresponding to amino acids 148–161 has been reported to neutralize LPS (Little et al., 1994; Jiang et al., 2004). Peptides that contain fused sequences of amino acid regions corresponding to different functional domains within BPI show both bactericidal and LPS-neutralizing activity (Gray and Haseman, 1994). Thus, a peptide corresponding to human BPI amino acids 90–99 and 148–161 was synthesized to evaluate whether the fusion of sequences of amino acid regions from two functional domains of BPI retained bifunctionality and exerted bactericidal activity against various bacterial species isolated from clinical cases of bovine mastitis.

3.2. Susceptibility of clinical mastitis bacterial isolates to the bacteriostatic and bactericidal effects of BPI_{pep}

The broth microdilution susceptibility assay was used to assess the spectrum of activity of BPI_{pep} against various Gram-negative bacteria isolated from clinical cases of mastitis (Table 1). The range of MIC and MBC values from six assays are shown. With the exception of *S. marcescens*, all species were susceptible to the bacteriostatic and bactericidal effects of BPI_{pep}. The peptide's MIC values were $\leq$64 μg/ml for all *E. coli* isolates, and $\leq$128 μg/ml for five of the six isolates of *P. aeruginosa* and all isolates of *K. pneumoniae* and *Enterobacter*. At concentrations up to 512 μg/ml, BPI_{pep} had no demonstrable growth inhibitory effect on any of the *S. marcescens* isolates tested. Because others have reported that *S. marcescens* is resistant to the bactericidal effects of full-length human and rabbit BPI (Beckerdite et al., 1974; Levy et al., 2000), it is not surprising that this bacterium is resistant to a peptide derived from the human BPI protein.

Interestingly, the reference strain of *S. aureus* exhibited sensitivity to the bacteriostatic and bactericidal effects of BPI_{pep}. Although *S. aureus* is reportedly resistant to the bactericidal activity of the full-length BPI protein, peptides containing sequence corresponding to amino acids 90–99 of this protein have been demonstrated to be bactericidal against *S. aureus* (Gray and Haseman, 1994). Thus, the sensitivity of *S. aureus* to BPI_{pep} reported here is consistent with the findings of a previous study.

Table 1
Antimicrobial activity of a human BPI-derived peptide (BPI_{pep}) against clinical mastitis isolates of Gram-negative bacteria

	BPI_{pep}		Tetracycline	
	MIC	MBC	MIC	MBC
Enterobacter aerogenes				
Isolate #1	32–64	32–128	>128	>128
Enterobacter cloacae				
Isolate #1	64	64	>128	>128
Isolate #2	32–64	32–64	2–4	>128
Isolate #3	64–128	64–128	128	128
Isolate #4	32–64	32–128	4–16	>128
Escherichia coli				
Isolate #1	16	32	2	64–128
Isolate #2	16–32	16–32	2	>128
Isolate #3	32	32–64	2	>128
Isolate #4	32–64	32–64	2	>128
Isolate #5	32–64	32–128	2	64–128
Strain P4	32–64	64–128	1–2	>128
ATCC 25922	32–64	32–64	0.5–1	32–128
Klebsiella pneumoniae				
Isolate #1	32–64	32–64	64–128	128
Isolate #2	32–64	32–64	128	128
Isolate #3	64–128	64–128	2	128
Isolate #4	64–128	64–128	64–128	128
Isolate #5	32–64	32–64	2	128
Isolate #6	32–64	32–64	1–16	128
Pseudomonas aeruginosa				
Isolate #1	64–128	64–128	16	>128
Isolate #2	64–128	64–128	8–16	>128
Isolate #3	64–128	64–128	64	128
Isolate #4	64–128	64–256	16–32	>128
Isolate #5	128–256	128–256	32	>128
Isolate #6	64–128	64–128	16–32	>128
Serratia marcescens				
Isolate #1	>512	>512	32–64	>128
Isolate #2	>512	>512	64	>128
Isolate #3	>512	>512	64	>128
Isolate #4	>512	>512	64	>128
Isolate #5	>512	>512	64	>128
Isolate #6	>512	>512	64	>128
Staphylococcus aureus				
ATCC 29213	128–256	128–256	<0.25–1	128 to >128

Minimum inhibitory (MIC) and bactericidal concentrations (MBC) are reported as μg/ml. The range of values from six broth microdilution assays evaluating peptide antimicrobial activity is shown.

The MBC values of BPI_{pep} were equivalent to or 1-fold higher than the range of MIC values for each susceptible strain (Table 1). For comparison, each bacterial isolate was also evaluated for its susceptibility to the bacteriostatic antibiotic, tetracycline. Within the concentration range tested, tetracycline inhibited the growth of almost all clinical mastitis isolates. Tetracycline MIC values for the two reference strains, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213, were within the established quality control ranges of 0.5–2 and 0.12–1 μg/ml, respectively (NCCLS, 1999). With the exception of two isolates of *Enterobacter* spp. and one isolate of *K. pneumoniae*, the MIC values of tetracycline were lower than that of BPI_{pep}. The MBC values of tetracycline were generally higher than those of BPI_{pep}. Thus, in contrast to the approximate 1:1–2:1 MBC:MIC ratios of BPI_{pep}, the ratio of tetracycline MBC values relative to those of its MIC were higher. These data are consistent with the bacteriostatic nature of tetracycline and the bactericidal nature of BPI.

3.3. Effect of amino acid substitutions and changes in sequence order on the bacteriostatic and bactericidal effects of BPI_{pep}

To determine whether the functionality of this 24-mer peptide was dependent upon specific amino acid composition or order, the antimicrobial activity of BPI_{pep} and three control peptides against the *E. coli* P4 strain were evaluated in parallel in the broth microdilution susceptibility assay (Table 2). To rule out that any random 24-mer peptide synthesized at comparable purity to that of BPI_{pep} could exert antimicrobial activity, a control peptide ($Ctrl_{pep1}$) was designed with an amino acid composition devoid of any sequence identity to BPI_{pep}. In contrast to BPI_{pep}, which displayed MIC's and MBC's as low as 16 and 64 μg/ml, respectively, $Ctrl_{pep1}$ did not demonstrate any growth inhibitory or bactericidal activity at concentrations $\leq$1024 μg/ml.

BPI_{pep}, similar to the whole BPI protein and other peptides with activity against Gram-negative bacteria, contains a high proportion of basic amino acids (Blondelle and Houghten, 1992; Nicolas and Mor, 1995; Pazgier et al., 2007). To determine whether the highly basic composition of BPI_{pep} was solely responsible for its activity, a second control peptide ($Ctrl_{pep2}$) was synthesized to contain alanine amino acids substituted for two corresponding tryptophans and one corresponding leucine within BPI_{pep}. Although this peptide contained the same proportion of basic and non-polar amino acids as BPI_{pep}, $Ctrl_{pep2}$ did not demonstrate any antimicrobial activity at concentrations $<$1024 μg/ml (Table 2). Thus, the antimicrobial activity of BPI_{pep} cannot be solely ascribed to its high content of basic amino acids.

Finally, to determine whether the specific order of amino acids within BPI_{pep} influenced activity, a third control peptide ($Ctrl_{pep3}$) was designed so that the stretch of amino acids corresponding to amino acids 90–99 within BPI protein (*i.e.*, the first 10 amino acids in BPI_{pep}) were placed after the amino acids corresponding to amino acids 148–161 within BPI (*i.e.*, the last 14 amino acids in BPI_{pep}). Reversing the order of these two stretches of amino acids decreased peptide activity, and corresponding MIC and MBC values were approximately 3–7 and 1–3-fold higher, respectively, than BPI_{pep}. Together, these data establish that the order and composition of amino acids within BPI_{pep} specifically influence antimicrobial activity of the peptide.

Table 2
Antimicrobial activity of a human BPI-derived peptide and various control peptides against *Escherichia coli* strain P4

Peptide designation	Modification	Sequence	MIC[a]	MBC
BPI_{pep}	–	KWKAQKRFLKKSKVGWLIQLFHKK	16–32	64
$Ctrl_{pep1}$	Unrelated sequence	MCHWAGGASNTGDARGDVFGKQAG	>1024	>1024
$Ctrl_{pep2}$	Alanine substitutions[b]	K**A**KAQKRFLKKSKVG**A**LIQ**A**FHKK	≥1024	≥1024
$Ctrl_{pep3}$	Reversed domains[c]	*KSKVGWLIQLFHKK***KWKAQKRFLK**	128	128–256

[a] Minimum inhibitory (MIC) and bactericidal concentrations (MBC) are reported as μg/ml. The range of values from four broth microdilution assays evaluating peptide antimicrobial activity is shown.
[b] Amino acids within BPI_{pep} substituted with alanine are bolded and underlined.
[c] Reversal of the order of the two functional domains within BPI_{pep} are indicated with different font styles.

3.4. Comparative bactericidal activity of BPI_{pep} in physiological fluids

To determine whether BPI_{pep} retained bactericidal activity in physiological fluids, the peptide was incubated with *E. coli* strain P4 (1×10^5 CFU/ml) in broth, serum or milk for 6 h (Table 3). This strain was chosen for evaluation because of its origin from a clinical case of mastitis and well-characterized pathogenesis (Bramley, 1976; Anderson et al., 1977; Bannerman et al., 2004). At concentrations $\geq$10 μg/ml, BPI_{pep} demonstrated bactericidal activity against *E. coli* in both broth and serum. In milk, however, the peptide demonstrated no bactericidal activity, even at a concentration that was 100× the effective dose in broth and serum. BPI_{pep} did exert a bacteriostatic effect in milk at a concentration of 1000 μg/ml and inhibited bacterial growth by >99%.

To assess whether components in a particular fraction of milk might be responsible for the inhibition of BPI_{pep} activity, the peptide was evaluated in whey, which is the protein-rich liquid fraction of milk devoid of cells, casein, and fat (Table 3). At 10 and 100 μg/ml, respectively, the peptide exhibited complete growth inhibitory and bactericidal activity against *E. coli*.

3.5. Effect of EDTA on BPI_{pep} activity in milk

Divalent cations are known to impair the activity of and/or sensitivity to various antibiotics (Russell, 1967; Miles and Maskell, 1986; Miles et al., 1986). Magnesium and calcium, which are present at high concentrations in milk, have been reported to inhibit the activity of the full-length BPI protein (Weiss et al., 1975, 1978). To determine whether high cation concentrations contribute to the loss of BPI_{pep} activity in milk, increasing concentrations of the divalent chelator EDTA were added to milk and BPI_{pep} antimicrobial activity assayed (Fig. 1). In the absence of BPI_{pep}, EDTA demonstrated no growth inhibitory effect on *E. coli* at concentrations $\leq$8 mM. The bacterial growth in milk during the 6 h incubation period, from a starting inoculating concentration of

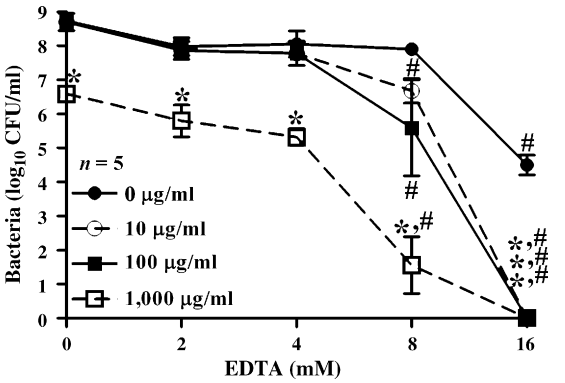

Fig. 1. Effect of EDTA on the bacteriostatic and bactericidal activity of a human BPI-derived peptide (BPI_{pep}) in milk. Various concentrations of BPI_{pep} and/or EDTA were added to milk containing 1×10^5 CFU/ml of *E. coli* strain P4. The inoculated milk was then incubated for 6 h at 37 °C, and subsequently serially diluted and plated. On the following day, colonies were enumerated and the mean (±S.E.) $\log_{10}$ CFU/ml calculated (S.E. bars smaller than the size of corresponding symbols are not shown). For each concentration of EDTA, the asterisk (*) denotes a significant difference ($P < 0.05$) relative to inoculums grown in the same concentration of EDTA in the absence of BPI_{pep} (*i.e.*, 0 μg/ml; solid line with closed circles). For each concentration of BPI_{pep}, the pound sign (#) denotes a significant difference ($P < 0.05$) relative to inoculums exposed to the same concentration of BPI_{pep} in the absence of EDTA (*i.e.*, 0 mM).

Table 3
Antimicrobial activity of a human BPI-derived peptide (BPI_{pep}) against *Escherichia coli* strain P4 in various biological fluids

BPI_{pep} (μg/ml)	Broth			Serum			Milk			Whey		
	Mean	S.E.	*n*	Mean	S.E.	*n*	Mean	S.E.	*n*	Mean	S.E.	*n*
0	8.90	0.02	5	8.20	0.05	8	8.96	0.08	8	8.81	0.08	8
0.01	8.85	0.03	5	8.21	0.04	8	N.D.	–	–	N.D.	–	–
0.1	8.68	0.04	5	8.17	0.03	8	N.D.	–	–	8.71	0.21	8
1	3.56	0.97	5	7.90	0.14	8	N.D.	–	–	8.05	0.26	8
10	0	0	5	0	0	8	8.95	0.09	8	4.85	0.2	8
100	0	0	5	0	0	8	7.60	0.18	8	0	0	8
1000	N.D.	–	–	N.D.	–	–	6.67	0.042	8	0	0	8

Mean (±S.E.) $\log_{10}$ colony forming units are reported. *n*: number of replicates. N.D.: not determined

1×10^5 CFU/ml to a final concentration of 8.14×10^8 CFU/ml, was completely inhibited in the presence of 16 mM EDTA. Consistent with results shown in Table 3, BPI_{pep} at a concentration of 1000 μg/ml demonstrated growth inhibitory activity in milk even in the absence of EDTA (*i.e.*, 0 mM). In the presence of 16 mM EDTA, all concentrations of BPI_{pep} demonstrated bactericidal activity in milk. At the highest concentration of EDTA that did not exert a bacteriostatic effect (*i.e.*, 8 mM), all three concentrations of BPI_{pep} demonstrated growth inhibitory activity and those ≥100 μg/ml inhibited >99% of *E. coli* growth in milk. These findings suggest that cations contribute to the impairment of BPI_{pep} antimicrobial activity in milk.

3.6. Effect of cations on BPI_{pep} activity in whey

As shown in Table 3, BPI_{pep} demonstrated bactericidal activity in whey, but not in milk. Approximately 25 and 65%, respectively, of the total magnesium and calcium content of milk is associated with casein (Fransson and Lonnerdal, 1983; Gaucheron, 2005; Neville, 2005). Whey, which is depleted of casein, contains correspondingly reduced levels of these cations. Thus, it could be hypothesized that BPI_{pep} retains its antimicrobial activity in whey due to the lower concentrations of cations that are present in whey. To determine whether increasing the cation concentration in whey could impair BPI_{pep} activity, the antimicrobial effects of BPI_{pep} against *E. coli* strain P4 was assayed in whey spiked with increasing concentrations of $MgCl_2$ or $CaCl_2$ (Fig. 2). Relative to activity in normal (unspiked) whey, BPI_{pep} demonstrated reduced activity in whey spiked with 3 mM Mg^{2+} or Ca^{2+}. These data are consistent with those reported in a previous study demonstrating inhibition of the antimicrobial activity of the whole human BPI protein in the presence of 2.5 mM Mg^{2+} or Ca^{2+} (Weiss et al., 1978). In the presence of 30 mM Ca^{2+}, BPI_{pep} lost all growth inhibitory activity in whey (Fig. 2B). Because the reported concentrations of Mg^{2+} and Ca^{2+} in milk range from 4 to 6 and 26 to 32 mM (Gaucheron, 2005), respectively, these data demonstrate that BPI_{pep} loses its activity in whey supplemented with concentrations of magnesium or calcium approaching those in milk. This finding, in combination with data demonstrating that chelation of

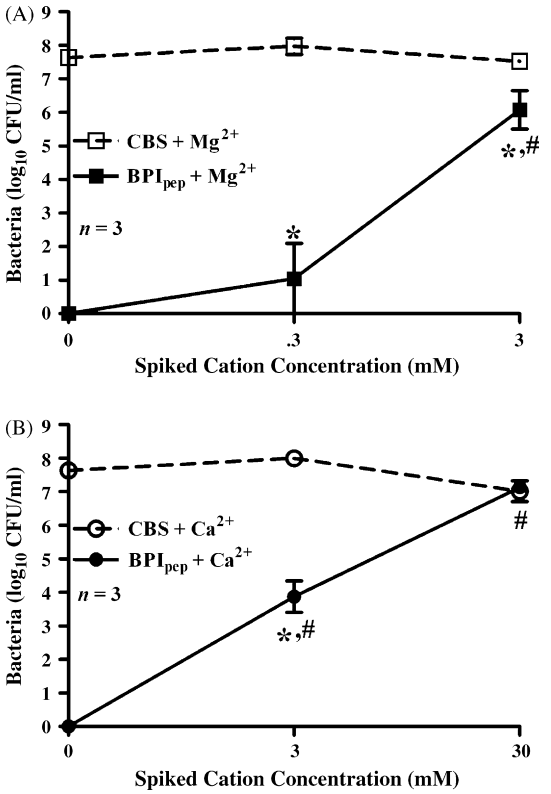

Fig. 2. Effect of cations on the bacteriostatic and bactericidal activity of a human BPI-derived peptide (BPI_{pep}) in whey. BPI_{pep} (800 μg/ml) or citrate-buffered saline (CBS) were added to whey containing 1×10^5 CFU/ml of *E. coli* strain P4 and spiked with varying concentrations of $MgCl_2$ (A) or $CaCl_2$ (B). The mixture was incubated for 6 h at 37 °C, and subsequently serially diluted and plated. On the following day, colonies were enumerated and the mean (±S.E.) $\log_{10}$ CFU/ml calculated (S.E. bars smaller than the size of corresponding symbols are not shown). *Significantly ($P < 0.05$) decreased relative to inoculums grown in CBS at the same concentration of spiked cation. #Significantly ($P < 0.05$) increased relative to inoculums exposed to BPI_{pep} in the absence of spiked cations (*i.e.*, 0 mM).

cations enhances BPI_{pep} activity in milk, suggests that the differential activity of BPI_{pep} in whey and milk can be attributed, in part, to differences in cation concentrations.

In addition to the potential inhibitory roles of divalent cations to inhibit BPI_{pep} activity in milk, one cannot exclude the possibility that other components are capable of sequestering BPI_{pep} and restricting it from contacting the bacterial cell wall. Milk contains an abundance of sialoglycoconjugates, including sialic acid bound oligosaccharides and glycoproteins

(Mather and Keenan, 1975; Martin et al., 2001). Both BPI_{pep} and the mature, native form of BPI are basic molecules with p*I*'s of 11.51 and 9.44, respectively; therefore, ionic interactions between sialic acid and these molecules are possible. Such interactions could impair the therapeutic efficacy of recombinant BPI and/or BPI-derived peptides in milk and may also impede endogenous neutrophil-derived BPI from exerting its bactericidal activity against intramammary bacteria during mastitis.

3.7. LPS-neutralizing activity of BPI_{pep}

To determine whether BPI_{pep} could neutralize LPS at concentrations commensurate with those that were bactericidal, increasing concentrations of the peptide were incubated with LPS (1 ng) and the resulting mixture assayed in the LAL assay (Fig. 3). Three control peptides and polymyxin B, the latter of which is a potent neutralizer of LPS, were also evaluated. At concentrations of 10 and 30 μg/ml, respectively, BPI_{pep} inhibited 27 and 90% of LPS bioactivity (Fig. 3A). In the presence of 100 μg/ml of BPI_{pep}, LPS was completely neutralized. $Ctrl_{pep1}$, which lacks any sequence identity with BPI_{pep}, demonstrated no LPS-neutralizing activity even at 100 μg/ml. $Ctrl_{pep2}$, which contains alanine amino acids substituted for two corresponding tryptophans and one corresponding leucine within BPI_{pep}, neutralized <50% of LPS activity at the maximal concentration tested (*i.e.*, 100 μg/ml). At concentrations ranging from 1 to 30 μg/ml, $Ctrl_{pep2}$ inhibited LPS bioactivity by ∼15%. This effect appeared to be non-specific as there was no concentration-dependent change in $Ctrl_{pep2}$ activity within this concentration range. $Ctrl_{pep3}$, whose sequence corresponded to the last 14 amino acids within BPI_{pep} followed by the first 10 amino acids within BPI_{pep}, demonstrated reduced LPS-neutralizing activity compared with BPI_{pep}. At 10 and 30 μg/ml, $Ctrl_{pep3}$ inhibited 1 and 33% of LPS bioactivity. At 100 μg/ml, $Ctrl_{pep3}$ neutralized 87% of LPS activity.

Polymyxin B inhibited LPS activity in a dose-dependent manner and neutralized >90% of LPS bioactivity at concentrations ≥10 μg/ml. On a raw mass/volume basis, a concentration of BPI_{pep} 2× higher than that of polymyxin B was necessary to achieve comparable neutralization. On a molar basis, however, a BPI_{pep} concentration (9.91 μM) only

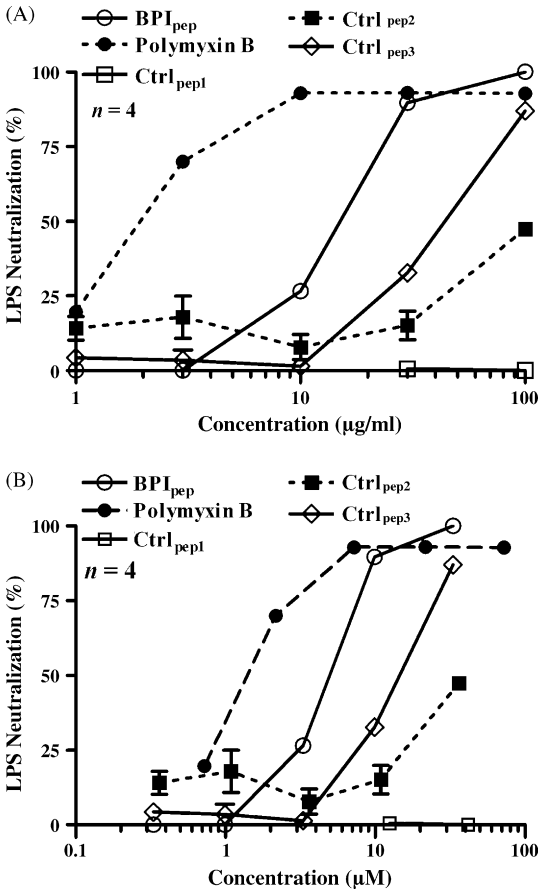

Fig. 3. LPS-neutralizing activity of a peptide derived from human BPI (BPI_{pep}). *E. coli*-derived LPS (1 ng) was incubated with various concentrations of BPI_{pep}, control peptides, or polymyxin B for 30 min. Following incubation, the amount of free LPS was determined with the *Limulus* amebocyte lysate assay and the mean (±S.E.) percent of bound (neutralized) LPS calculated (S.E. bars smaller than the size of corresponding symbols are not shown). Concentrations of tested agents (*x*-axis) are shown in μg/ml (A) and μM (B) units on a log scale.

0.37× higher than that of polymyxin B (7.22 μM) was necessary to achieve 90% inhibition of LPS bioactivity (Fig. 3B). The effective molar concentrations at which polymyxin B, BPI_{pep}, and $Ctrl_{pep3}$ could inhibit 50% of LPS bioactivity were 1.6, 5.8, and 17 μM, respectively. At the concentrations tested, $Ctrl_{pep1}$ and $Ctrl_{pep2}$ were unable to inhibit 50% of activity.

In terms of bifunctional capability to neutralize LPS and kill bacteria, BPI_{pep} exhibited both effects at concentrations <100 μg/ml. This combined activity

of the peptide indicates its potential for therapeutic application to the treatment Gram-negative infections. Currently approved antibiotics for systemic use only target the pathogen and do not counteract the LPS molecule, the latter of which is responsible for evoking the excessive inflammatory response that threatens the life of the animal. BPI_{pep}, however, has the ability to target both the pathogen and LPS.

The demonstrated bactericidal activity of BPI_{pep} in serum suggests that additional studies investigating its therapeutic potential for the treatment of septicemia are warranted. The impaired bactericidal activity of BPI_{pep} in milk may diminish interest in further evaluating its therapeutic potential for the treatment of intramammary infections. However, the cations that may be responsible for the impairment of BPI_{pep} activity in normal milk are reported to decrease by $>50\%$ during mastitis (Bogin and Ziv, 1973; El Zubeir et al., 2005). Thus, changes in milk composition during the course of mastitis may enable BPI_{pep} to have a therapeutic benefit in the treatment of this disease.

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