Zinc potentiates the antibacterial effects of histidine-rich peptides against *Enterococcus faecalis*

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Running title: Antibacterial histidine-rich peptides

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Abbreviations

HMWK, high molecular weight kininogen; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Keywords

antimicrobial peptide; high molecular weight kininogen; *Enterococcus faecalis*; zinc; heparin

Antimicrobial peptides are effector molecules of the innate immune system. We recently showed that peptides containing multiples of the heparin-binding Cardin and Weintraub motifs AKKARA and ARKKAAKA exert antimicrobial activities. Here, we show that the replacement of lysine and arginine in these motifs by histidine, abrogates the antibacterial effects of these peptides. Antibacterial activity of the histidine-rich peptides against the Gram-positive bacterium *Enterococcus faecalis* was restored by addition of Zn^{2+}. Fluorescence microscopy experiments showed that Zn^{2+} enabled binding of the histidine-rich peptides to *E. faecalis* bacteria. Similar Zn^{2+}-dependent antibacterial activities were demonstrated for histatin 5 as well as histidine-containing peptides derived from the Zn^{2+} and heparin-binding domain 5 of human kininogen. Thus, the results demonstrate a previously undisclosed Zn^{2+}-dependent antibacterial activity of kininogen-derived peptides and indicate an important role for Zn^{2+} in the regulation of antimicrobial activities of histidine-rich peptides.

Introduction

Antimicrobial substances in blood and leukocytes were discovered over 100 years ago (for review, see [1]). The identification of antimicrobial peptides (AMPs) in polymorphonuclear leukocytes [2] was followed by a molecular characterization of these molecules [3, 4]. The subsequent discovery of AMPs in invertebrates [5] and cold-blooded vertebrates [6], has emphasized the evolutionary importance of this group of host defence molecules. At present, over 800 different AMP peptide sequences are known (see www.bbcm.univ.trieste.it/~tossi/search.htm). Many AMPs adopt an amphipathic structure, where clusters of hydrophobic and cationic amino acids are spatially organized in sectors of the molecules. Thus, peptides may be grouped into linear peptides which adopt an α-helical and amphipathic conformation upon entering a bacterial membrane, peptides composed of

cysteine-linked antiparallel β-sheets, peptides with a cysteine-constrained loop structure, or peptides with an over-representation of some amino acids [7, 8]. It is well established that bacterial binding and thus, interaction with bacterial membranes is a prerequisite for AMP-function. However, the modes of action of AMPs on their target bacteria are complex, and can be divided into membrane and non-membrane disruptive. With respect to amphipathic and α-helical AMPs, such as the human cathelicidin LL-37**,** these AMPs are able to interact with bacterial surface components such as lipopolysaccharide and peptidoglycans, leading to induction of an α-helical conformation, which in turn facilitates membrane interactions, membrane destabilization and finally, bacterial killing [9]. In contrast, other AMPs, such as the porcine cathelicidin PR-39 and human histatins function by less well elucidated mechanisms. Whereas PR-39 blocks bacterial DNA and protein synthesis [10], histatins translocate through membranes [11] and bind to a receptor in the fungal mitochondrion, whereby they may induce cell death by non-lytic ATP release, generation of reactive oxygen species and induction of G1 phase arrest [12, 13]. Apart from their antimicrobial activities, AMPs also interact with negatively charged glycosaminoglycans, including heparin [14]. Conversely, we recently showed that several naturally occurring cationic peptide segments with heparin-binding capabilities, including the anaphylatoxin C3a and histidine and lysine-rich peptides of domain 5 of human high molecular weight kininogen (HMWK), were antimicrobial [15, 16]. In conjunction with these findings, consensus heparin-binding peptide sequences (Cardin and Weintraub motifs) XBBBXXBX or XBBXBX (where X represents hydrophobic or uncharged amino acids, and B represents basic amino acids), represented by multiples of the motifs ARKKAAKA or AKKARA [17], were shown to be antibacterial against the Gram-positive bacterium *Enterococcus faecalis* and the Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli* [18]. The starting point for this study was the observation that histidine-rich peptides, such as those derived from the histidine and glycine-

rich domain 5 of HMWK require Zn^{2+} for interaction with heparin. Here we show that these kininogen-derived peptides, as well as prototypic histidine-rich Cardin and Weintraub peptides are antimicrobial in presence of Zn^{2+}, thus disclosing an interesting role for this divalent cation in the regulation of antimicrobial activities of histidine-rich peptides.

Results

Antimicrobial activities of histidine-rich peptides containing heparin-binding motifs

As previously shown, Cardin and Weintraub motif peptides $(AKKARA)_4$ (AKK24) and $(ARKKAAKA)_3$ (ARK24) (Table 1) interact with heparin [17] and exert antimicrobial effects mediated by disruption of bacterial membranes [18]. In order to study the antimicrobial as well as heparin-binding activities of corresponding histidine-substituted peptide motifs, peptides with amino acids R and K replaced by H were synthesized, thus yielding the sequences $(AHHAHA)_4$ and $(AHHHAAHA)_3$, denoted AHH24:1 and AHH24:2, respectively (Table 1). For screening of heparin-binding, an established slot-binding assay was used [17]. The results showed that the 24-amino acid histidine-rich amphipathic peptides displayed a weak binding to radiolabelled heparin in 10 mM Tris, pH 7.4. Addition of 50 µM Zn^{2+} to the buffer increased the heparin-binding (Fig. 1A, upper panel). In contrast, the AKK24 and ARK24 peptides bound heparin in absence and presence of Zn^{2+} (Fig. 1A, lower panel). Next, we investigated the effects of these peptides on bacteria, and specifically, we wanted to study potential enhancement of peptide antibacterial activities by Zn^{2+}. As previous reports have demonstrated that Zn^{2+} may exert antibacterial effects *per se* [19], we first screened various Gram-positive and Gram-negative bacteria for susceptibility to Zn^{2+}. As shown in Table 2, among the bacteria tested, the Gram-positive *E. faecalis* demonstrated least sensitivity to

Zn^{2+}, and was therefore selected for furher analyses. Thus, *E. faecalis* 2374 bacteria were incubated with the lysine and arginine-containing peptides AKK24 and ARK24, or the two histidine-containing peptides AHH24:1 and AHH24:2. Whereas AKK24 and ARK24 effectively killed the bacteria at ~1 μM, little or no antibacterial effects of the AHH-peptides were detected in the Tris buffer at pH 7.4 (Fig. 1B). However, both the AHH peptides exerted antibacterial effects in the presence of 50 μM Zn^{2+}. At this Zn^{2+} concentration, an antibacterial effect was noted at peptide concentrations of 0.1-1 μM. However, we repetitively noted a diminished antibacterial activity at peptide concentrations of 3-6 μM (molar Zn^{2+} to peptide ratio of ~10). Hypothetically, this could be due to Zn^{2+}-peptide complex formations, or peptide oligomerisations at certain threshold levels of Zn^{2+} to AHH peptide, leading to inhibition of peptide interactions with bacterial membranes. The findings that the inhibition was abolished at higher peptide concentrations (Fig. 1B), as well as using a constant molar excess of Zn^{2+} to peptide (Fig. 1C), is compatible with this hypothesis. In contrast to the experiments with the AHH-peptides, the AKK24 and ARK24 peptides displayed no enhancement of antibacterial activities in presence of Zn^{2+} (Fig. 1B). Next, we investigated the effect of the peptides AHH24:1 and AHH24:2 against different strains of *E. faecalis* in buffer with or without 50 μM Zn^{2+}. The results showed that the strains were sensitive to 100 μM AHH-peptides in presence of 50 μM Zn^{2+} (Fig. 1D).

Analysis of peptide binding to bacterial membranes and effects of ions

To examine whether the AHH-peptides interact with bacterial membranes, AHH24:1 and AHH24:2 were labelled by the fluorescent dye Texas Red and incubated with *E. faecalis* 2374 bacteria in absence or presence of 50 μM Zn^{2+}. As demonstrated by fluorescence microscopy analysis, the peptides bound to the bacteria in presence of Zn^{2+} (Fig. 2A). Furthermore, the binding was completely blocked by heparin, thus indirectly demonstrating the heparin-binding

capability of these peptides. Heparin did not quench the fluorescence of the Texas Red labelled peptides (not shown). Having shown a prerequisite for Zn^{2+} for bacterial killing, we analysed the influence of Mg^{2+} and Ca^{2+} on the antibacterial activities of the AHH-peptides. As demonstrated in Fig. 2B, only Zn^{2+} significantly increased bacterial killing.

Antimicrobial activities of peptides derived from HMW-kininogen

The domain 5 of HMWK contains two subdomains. One domain is His-Gly-rich (K420-D474) and one His-Gly-Lys-rich (G474-K502). As shown by Pixley *et al* [20], the heparin-binding activity of the His-Gly-rich domain is Zn^{2+} dependent whereas the His-Gly-Lys-rich domain binds heparin independently of Zn^{2+} [20]. As previously shown, a peptide from the latter domain (HKH20, Table 1, Fig. 3A) binds to heparin in absence of Zn^{2+} [16] (here shown for completeness in Fig. 3B, upper panel) and exerts potent antibacterial effects independently of Zn^{2+} [16]. Here, additional peptides spanning domain 5 (Fig. 3A) were tested for heparin-binding as well as for antibacterial activity in absence or presence of Zn^{2+}. The peptides KHN20 (K420-R439) and GGH20 (G469-H488) demonstrated no or weak binding to heparin in absence of Zn^{2+} (Fig. 3B, upper panel). However, addition of Zn^{2+} yielded enhanced binding to heparin for these peptides (Fig 3B, lower panel). The peptides GHG20 and GHG21 (G440-F459 and G454-D474 respectively), derived from a region of low heparin affinity [20] did not bind to heparin in our screening assay, and the binding was not enhanced by Zn^{2+} (not shown). Antibacterial assays demonstrated that Zn^{2+} potentiated the antibacterial activity of the peptides KHN20 and GGH20 (Fig. 3C), which paralleled the results obtained from the heparin-binding assay (Fig. 3B, lower panel).

Antimicrobial activities of histatin 5 in presence of ions

Finally, we investigated the antibacterial effect of the histidin-rich peptide histatin 5 against

E. faecalis 2374 in presence of different ions. The results showed that Zn^{2+} significantly potentiated the antimicrobial activity of histatin 5 (Fig. 4A). As demonstrated in Fig. 4B, Zn^{2+} and Ca^{2+}, but not Mg^{2+}, were able to increase the antibacterial activity of 0.3 μM histatin 5.

Discussion

Electrostatic and hydrophobic interactions of lysine and arginine-rich amphipathic peptides mediate interactions with negatively charged bacterial membranes. The replacement of lysine and arginine residues in the AMPs AKK24 and ARK24 (Table 1) by histidines (yielding the AHH24 peptides, Table 1) completely abolished the antimicrobial capacity of these peptides. By imposing a positive charge on the AHH24 peptides, i. e. by addition of Zn^{2+}, which specifically binds to histidine-rich peptide regions, we were able to demonstrate a restored antibacterial activity of these motif peptides**,** and this activity corresponded with an ability to interact with heparin, a negatively charged glycosaminoglycan. The demonstration that Zn^{2+} potentiated the antimicrobial activity of a set of peptides from the heparin and Zn^{2+}-binding regions of domain 5 of HMWK, further substantiate the findings with the prototypic AHH-peptides. Whether similar peptides of the His-Gly and Zn^{2+} dependent domain of HMWK are generated during proteolysis, was not addressed in this study and remains to be investigated. However, domain 5-derived antibacterial fragments comprising the peptide HKH20, which exerts potent antimicrobial effects independent of Zn^{2+}, are generated after proteolysis of HMWK [16]. Apart from domain 5-derived AMPs, bradykinin of domain 4 was found to possess antimicrobial activities [21]. Furthermore, the vascular permeability enhancing peptide (E-kinin), SLMKRPPGFSPFRSSRI, containing the bradykinin peptide, generated by the concerted actions of mast cell tryptase and neutrophil elastase [22, 23], is also antimicrobial against both *P. aeruginosa* and *S. aureus* [16]. Thus, proteolytic degradation of

HMWK releases multiple AMPs, raising the possibility that Zn^{2+} dependent AMPs are also generated during this process.

At present, multiple histidine-rich AMPs are known, including histatins in human saliva [24], haebrein from the hard tick *Amblyomma hebraeum* [25], clavaspirin from the tunicate *Stylea clava* [26], and semenogelin-derived peptides from human semen [27]. Zn^{2+}, a physiologically significant cation influences crucial biological processes such as coagulation, contact activation, transcriptional control, and enzyme function, by binding to, and stabilizing various proteins including histidine-rich glycoprotein, kininogen, zinc-finger proteins, and various metalloproteinases. In line with reports indicating that histatin 5 specifically binds to Zn^{2+} [28], our study demonstrates that the antibacterial activity of histatin 5 is enhanced by Zn^{2+}, thus providing a further proof of the concept that Zn^{2+} may regulate the antimicrobial activity of histidine-rich AMPs. In this context, it is interesting to note that the total concentration of Zn^{2+} in plasma is 10-18 μM, but thrombocytes can accumulate levels of Zn^{2+} 25 to 60-fold higher than those found in plasma [29]. Furthermore, excessive Zn^{2+} levels are found in certain body compartments and organs. Thus, human skin has been reported to contain significant levels of Zn^{2+} (~0.5 mM) [30]. Likewise, the Zn^{2+} levels in semen are in the mM-range. Whether the histidine-rich semenogelins in human semen also exhibit similar Zn^{2+}-dependent activities remains to be investigated, however, it is of note that semenogelin binds to heparin and Zn^{2+} [31, 32]. In conclusion, our findings disclose a novel Zn^{2+} dependent antimicrobial activity for prototypic histidine-rich heparin-binding sequences, histatin 5 as well as HMWK-derived peptides, and point at an interesting role for Zn^{2+} in the control of antimicrobial activities of histidine-rich AMPs.

Experimental procedures

Materials

The peptides AKK24; $(AKKARA)_4$, ARK24; $(ARKKAAKA)_3$, AHH24:1; $(AHHAHA)_4$, AHH24:2; $(AHHHAAHA)_3$, KHN20, and GGH20 (see Table 1) and Texas red labelled peptides AHH24:1 and AHH24:2 were from Innovagen AB (Lund, Sweden). The purity and molecular weight was confirmed by MALDI-TOF MS analysis (Voyager, Applied Biosystems). Histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY) was kindly provided by Prof M. Malmsten, Uppsala University, Sweden.

The bacterial isolates *Enterococcus faecalis* 2374, BD 33/03, and BD 96/03, *Escherichia coli* 37.4 and 47.1, *Pseudomonas aeruginosa* 27.1 and 15159 were obtained from patients with chronic ulcers and *Staphylococcus aureus* 80 and BD 312 were from patients with atopic dermatitis. *E. faecalis* ATCC 29212, *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were obtained from The American Type Culture Collection (ATCC, Rockville, MD, USA).

Heparin-binding assay

The radioiodination of heparin (from porcine intestinal mucosa, Sigma-Aldrich, St Louis MO, USA) was performed according to previous protocols [33, 34]. 2 and 5 μg of the synthetic peptides were applied onto nitrocellulose membranes (Hybond-C, Hybond-C, GE Healthcare BioSciences, Little Chalfont, United Kingdom) using a slot blot apparatus. The membranes were incubated with radiolabelled heparin (~10 μg/ml, 0.4 x 10^6 cpm/μg) for 1 hour at room temperature in 10 mM Tris, pH 7.4 with or without 50 μM Zn^{2+}. The membranes were washed for 3 x 10 minutes in 10 mM Tris, pH 7.4. A Bas 2000 radio imaging system (Fuji) was used for visualization of radioactivity.

Viable count analysis

Bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium (Becton and Dickinson, Sparks, MD, USA) and washed in 10 mM Tris, pH 7.4. For analysis of effects of Zn^{2+} on bacterial survival, 50 μl bacterial suspension (containing ~1 x 10^5 cfu) was incubated in 10 mM Tris, pH 7.4 with 10, 25, 50, and 100 μM Zn^{2+}, plated on TH agar overnight at 37º C and the number of colony forming units (cfu) was determined. For analysis of the antibacterial activities of the peptides AHH24:1, AHH24:2, AKK24 and ARK24, or histatin 5 (Table 1), *E. faecalis* bacteria were incubated with the peptides ranging 0.03-60 μM for 2 hours in 10 mM Tris, pH 7.4 with or without 50 μM Zn^{2+}. In viable count assays using a fixed ratio of Zn^{2+}/peptide (Fig. 1C), *E. faecalis* 2374 bacteria (2 x 10^6/ml) were incubated together with the peptides AHH24:1 and AHH24:2 and the number of cfu was determined.

To determine the activity of AHH24:1 and AHH24:2 against different strains of *E. faecalis*, 100 μM AHH24:1 or AHH24:2 were incubated with *E. faecalis* 2374, *E. faecalis* BD 33/03, *E. faecalis* BD 96/03 or *E. faecalis* ATCC 29212 in 10 mM Tris, pH 7.4 in absence or presence of 50 μM Zn^{2+}. For analysis of effects of different ions, *E. faecalis* 2374 bacteria (2 x 10^6/ml) were incubated with 0.5 μM of AHH24:1, AHH24:2 or 0.3 μM histatin 5 in 10 mM Tris, pH 7.4 alone, or the same buffer containing 50 μM Zn^{2+}, 50 μM Mg^{2+} or 50 μM Ca^{2+}. In all experiments, 100% survival was determined as bacterial numbers obtained in absence of peptide in the corresponding buffer (with or without the respective ion). Significance was determined using Kruskall-Wallis one way ANOVA analysis, (SIGMASTAT, SPSS, Chicago IL, USA).

Fluorescence microscopy

E. faecalis 2374 bacteria were grown in TH medium at 37º C to mid-logarithmic phase. The bacteria were washed in 10 mM Tris, pH 7.4, and resuspended in the same buffer. One μl of *E. faecalis* (2×10^9 cfu/ml) were incubated with 2 μg of Texas Red labelled AHH24:1 or AHH24:2 in 10 mM Tris, pH 7.4 or 10 mM Tris, pH 7.4, 50 μM Zn^{2+}, with or without heparin (50 μg/ml, added to the peptides before addition to the bacteria) for 4 minutes on ice and subsequently washed twice in 10 mM Tris, pH 7.4. The bacteria were fixed with 4% paraformaldehyde, first on ice for 15 minutes and then in room temperature for 45 minutes, applied onto poly-L-lysine coated cover glass for 30 minutes and finally mounted on a slide by Dako mounting media (Dako, Carpinteria, CA, USA). For fluorescence analysis, bacteria were visualized using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with a Hamamatsu C4742-95 cooled CCD camera, a Plan Apochromat 100X objective and a high N.A. oil condenser. Differential interference contrast (Nomarski) imaging was used for visualisation of bacterial cells. Nomarski imaging is a modification phase microscopy where samples are visualized in phase microscopy by producing contrast from refractive index inhomogeneities rather than from light absorption inhomogeneities.

Acknowledgements

This work was supported by grants from the Swedish Research Council (projects 13471), the Royal Physiographic Society in Lund, the Welander-Finsen, Söderbergs, Crafoord, Österlund, and Kock Foundations, DermaGen AB, and The Swedish Government Funds for Clinical Research (ALF). We also wish to thank Ms. Mina Davoudi for expert technical assistance.

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Table 1. Peptides analysed in this study

Protein	Peptide	Sequence
Cardin motifs	AKK24	AKKARAAKKARAAKKARAAKKARA
	ARK24	ARKKAAKAARKKAAKAARKKAAKA
	AHH24:1	AHHAHAAHHAHAAHHAHAAHHAHA
	AHH24:2	AHHHAAHAAHHHAAHAAHHHAAHA
HMW kininogen	KHN20	KHNLGHGHKHERDQGHGHQR
	GHG20	GHGLGHGHEQQHGLGHGHKF
	GHG21	GHGHKFKLDDDLEHQGGHVLD
	GGH20	GGHVLDHGHKHKHGHGHGKH
	HKH20	HKHGHGHGKHKNKGKKNGKH
	Histatin-5	DSHAKRHHGYKRKFHEKHHSHRGY

Table 2. Effects of Zn^{2+} on various Gram-positive and Gram-negative bacteria. The indicated bacteria were incubated for 2 hours in 10 mM Tris, pH 7.4 alone, or in presence of Zn^{2+} at the indicated concentrations. After incubation, the number of cfu was determined. The numbers represent bacterial counts expressed in % relative the zinc-free control (defined as 100%). Standard deviations are indicated (n=3).

Gram-positive bacteria	**strain**	**10 μM Zn^{2+}**	**25 μM Zn^{2+}**	**50 μM Zn^{2+}**	**100 μM Zn^{2+}**
E. faecalis	2374	59.6 ± 6.7	69.3 ± 10.0	64.6 ± 6.4	45.2 ± 14.3
	BD 33/03	28.5 ± 3.8	28.9 ± 6.3	12.1 ± 2.7	9.4 ± 3.7
	ATCC 29212	78.1 ± 10.6	63.3 ± 9.9	56.0 ± 5.6	35.4 ± 8.3
S. aureus	80	0	0	0	0
	BD 312	0	0	0	0
	ATCC 29213	0	0	0	0
Gram-negative bacteria					
E. coli	37.4	0	0	0	0
	47.1	0	0	0	0
	ATCC 25922	0	0	0	0
P. aeruginosa	27.1	8.3 ± 7.5	1.1 ± 1.7	0	0
	15159	34.2 ± 8.1	20.7 ± 6.1	0	0
	ATCC 27853	0	0	0	0

Figure legends

Fig. 1. Heparin-binding and antibacterial effects of peptides containing Cardin and Weintraub motifs. (A) Slot-binding assay. Peptides (AHH24:1, AHH24:2, AKK24, and ARK24, at 2 and 5 μg) were applied to nitrocellulose membranes followed by incubation with iodinated (^{125}I) heparin in 10 mM Tris, pH 7.4 in absence (-) or presence of 50 μM Zn^{2+} (+). Radioactivity was visualized using a phosphorimager system. (B) Antibacterial assays. *E. faecalis* 2374 (2 x 10^6 cfu/ml) were incubated for 2 h at 37° C with the indicated peptides at concentrations ranging 0.03-60 μM in 10 mM Tris, pH 7.4 alone (●), or in presence of 50 μM Zn^{2+} (○), and the number of cfu was determined. (C) Antibacterial activities of the AHH24 peptides in presence of a fixed molar excess of Zn^{2+}. *E. faecalis* 2374 bacteria were incubated with AHH24:1 and AHH24:2 at the indicated concentrations in buffer containing a 100 times molar excess of Zn^{2+} (relative the peptide concentration). (D) Antibacterial effects of AHH24:1 and AHH24:2 against different strains of *E. faecalis*. In viable count assays, the indicated *E. faecalis* bacterial isolates were incubated with 100 μM of the AHH24 peptides in 10 mM Tris-buffer, pH 7.4 only (black bars) or in the same buffer containing 50 μM Zn^{2+} (white bars). Error bars indicate standard deviation (*** $p<0.001$, n=6).

Fig. 2. Binding of histidine-rich peptides containing Cardin and Weintraub motifs to bacteria and effects of divalent cations on bacterial killing. (A) Binding of Texas Red-labelled AHH24:1 and AHH24:2 peptides to *E. faecalis* 2374 bacteria in absence and presence of Zn^{2+} and inhibition of binding by an excess of heparin. *E. faecalis* bacteria were incubated with the indicated Texas Red-labelled AHH-peptides in 10 mM Tris buffer only (image set 1 and 4), Tris buffer with 50 μM Zn^{2+} (image set 2 and 5), or the same Zn^{2+} containing Tris buffer supplemented with heparin (50 μg/ml) (image set 3 and 6). The upper image row of images shows Nomarski images, whereas the lower image row shows red

fluorescence of bacteria. (B) Effects of divalent cations on peptide activity. *E. faecalis* 2374 were incubated with the peptides AHH24:1 and 24:2 peptides (at 0.5 μM) in presence of the indicated cations (all at 50 μM) and bacterial counts were determined. Bacterial numbers are expressed relative buffer controls containing the respective cations
The standard deviation is indicated by error bars (*** $p<0.001$, n=6).

Fig. 3. Activities of histidine-rich peptides derived from HMWK. (A) Sequence of domain 5 of HMWK and synthetic peptides used in the study are indicated. (B) Heparin-binding activity of the domain 5-derived peptides. Peptides at the indicated concentrations were applied to nitrocellulose membranes followed by incubation with (^{125}I) heparin in 10 mM Tris, pH 7.4 in absence (-) or presence of Zn^{2+} (+). The upper panel shows peptides (indicated in the figure) incubated in buffer in absence of Zn^{2+}. The lower panel shows effects of addition of 50 μM Zn^{2+} (+) to peptides KHN20 and GGH20. (C) In viable count assays, the effects of KHN20 and GGH20 were analysed. *E. faecalis* 2374 bacteria (2 x 10^6 bacterial cfu/ml) were incubated with the peptides KHN20 or GGH20 at concentrations ranging 0.03-60 μM in 10 mM Tris, pH 7.4 (●), or 10 mM Tris, pH 7.4 containing 50 μM Zn^{2+} (○).

Fig. 4. Antibacterial activity of histatin 5 and effects of ions. (A) In viable count assays, *E. faecalis* 2374 bacteria were incubated with histatin 5 at concentrations ranging 0.03-60 μM in 10 mM Tris, pH 7.4 (●), or the same buffer containing 50 μM Zn^{2+} (○), and the number of cfu was determined. (B) In viable count assays, *E. faecalis* 2374 bacteria were incubated with 0.3 μM histatin 5 for two hours in 10 mM Tris, pH 7.4 containing Zn^{2+}, Ca^{2+}, or Mg^{2+} (all at 50 μM), plated and the number of cfu was determined. Bacterial numbers are expressed relative buffer controls containing the respective cations**.** The standard deviation is indicated by error bars (*** $p<0.001$, n=6).

Figure 1

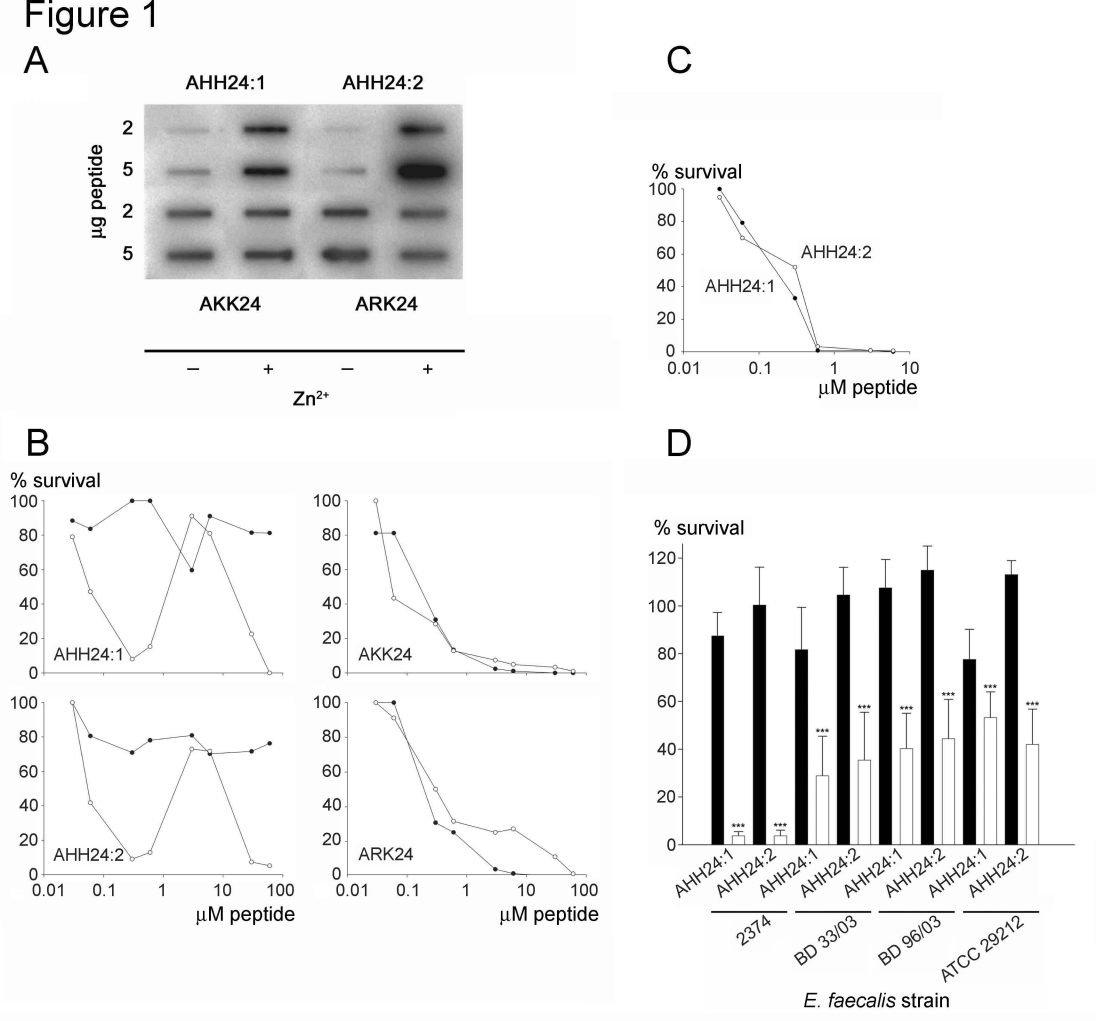
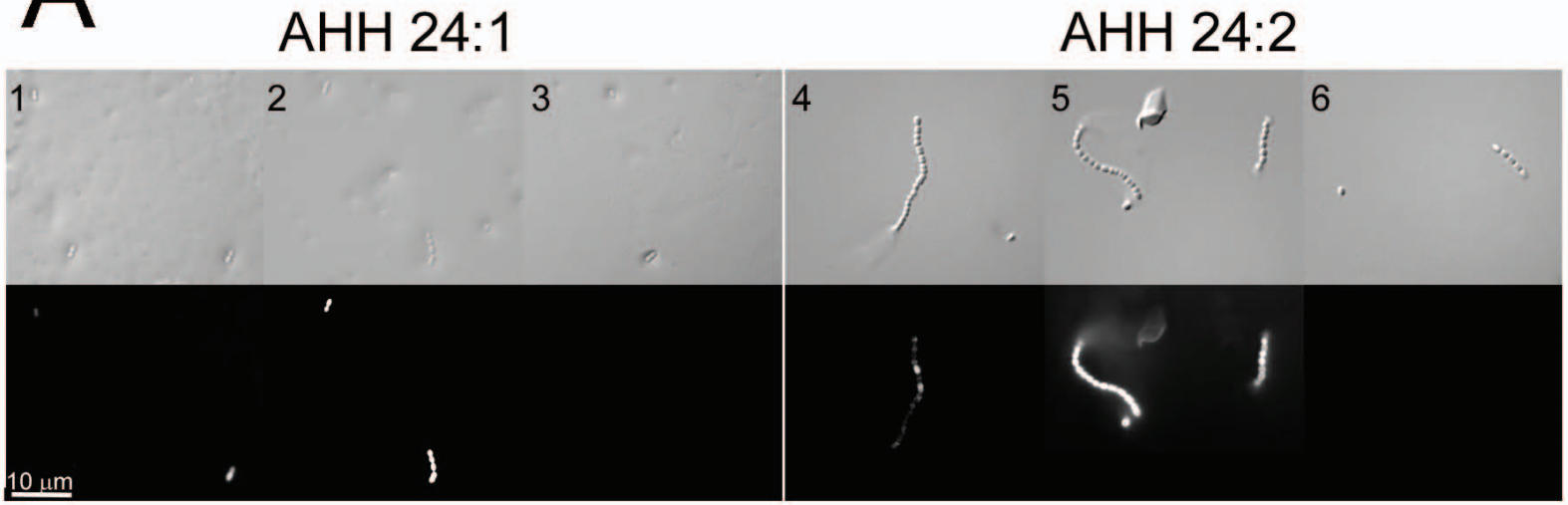

A
AHH24:1
AHH24:2
µg peptide
2
5
2
5
AKK24
ARK24
–
+
–
+
Zn2+
B
% survival
AHH24:1
AKK24
AHH24:2
ARK24
µM peptide
µM peptide
C
% survival
AHH24:2
AHH24:1
µM peptide
D
% survival
AHH24:1
AHH24:2
2374
BD 33/03
BD 96/03
ATCC 29212
E. faecalis strain

Figure 2

A


AHH 24:1
AHH 24:2
1
2
3
4
5
6
10 µm

B

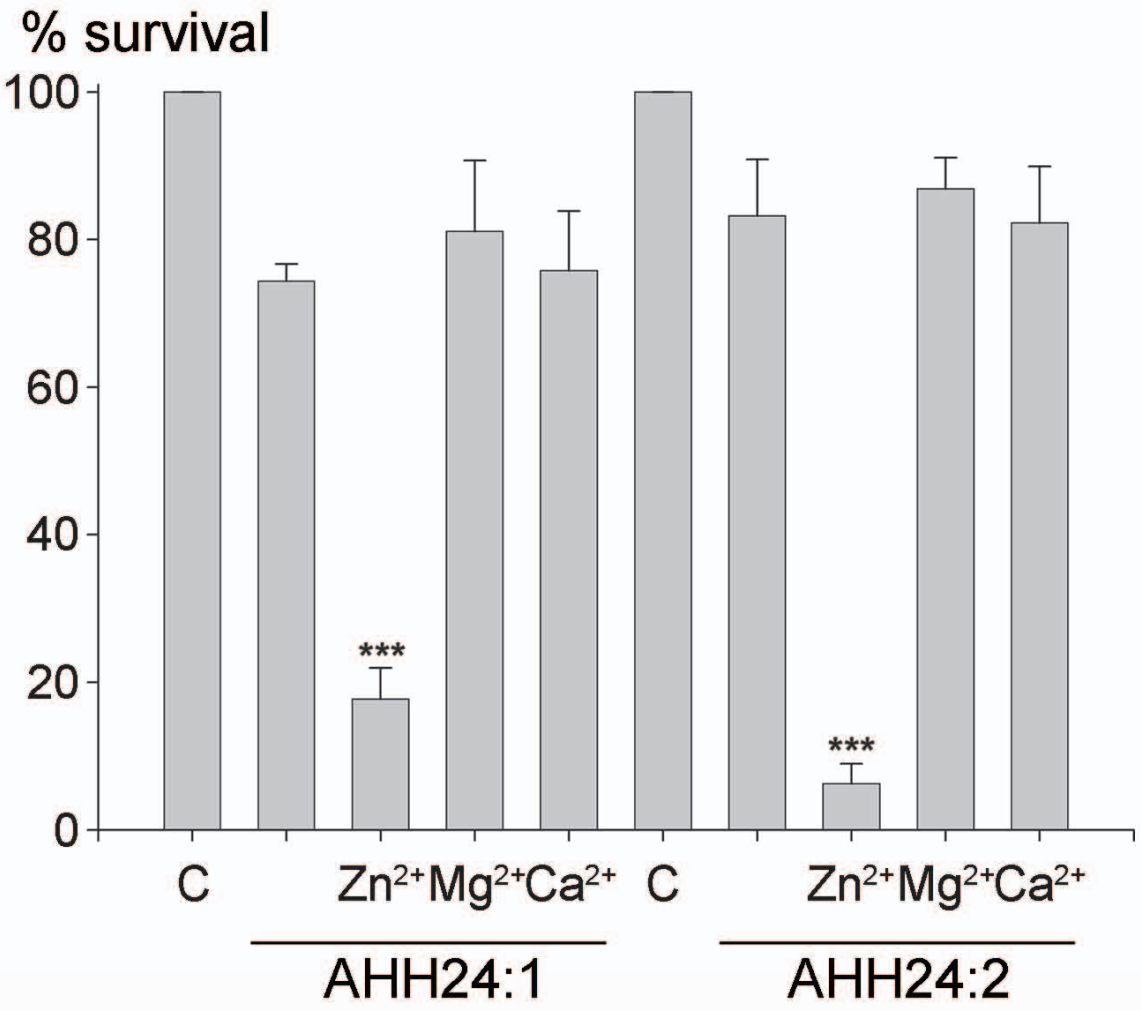
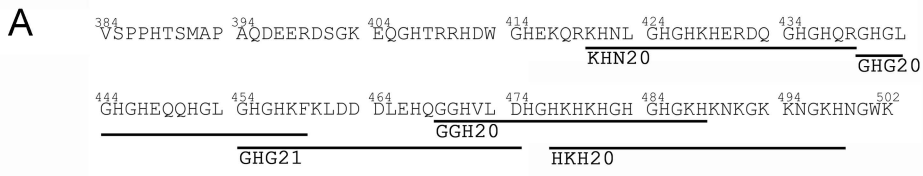
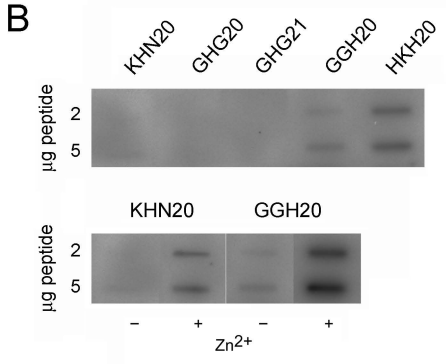

% survival
100
80
60
40
20
0
***
***
C
Zn2+Mg2+Ca2+
C
Zn2+Mg2+Ca2+
AHH24:1
AHH24:2

Figure 3


A
384 VSPPHTSMAP 394 AQDEERDSGK 404 EQGHTRRHDW 414 GHEKQRKHNL 424 GHGHKHERDQ 434 GHGHQRGHGL
KHN20
GHG20
444 GHGHEQQHGL 454 GHGHKFKLDD 464 DLEHQGGHVL 474 DHGHKHKHGH 484 GHGKHKNKGK 494 KNGKHNGWK 502
GGH20
GHG21
HKH20


B
KHN20
GHG20
GHG21
GGH20
HKH20
µg peptide
2
5
KHN20
GGH20
µg peptide
2
5
–
+
–
+
Zn2+

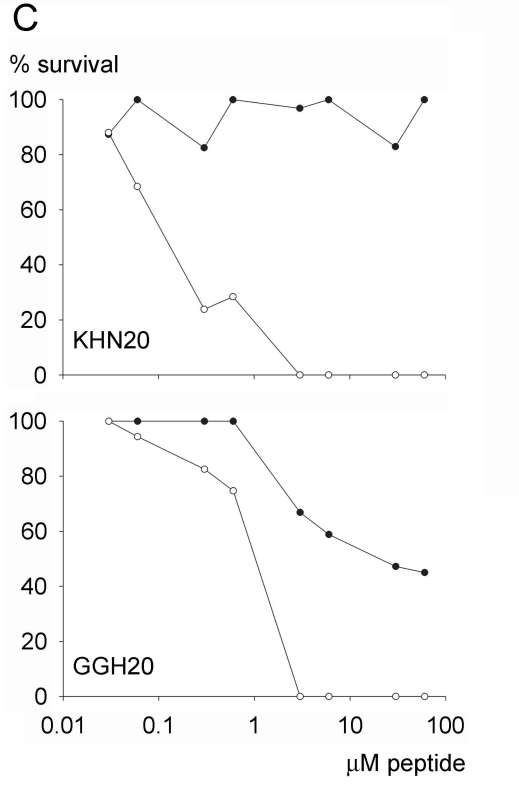
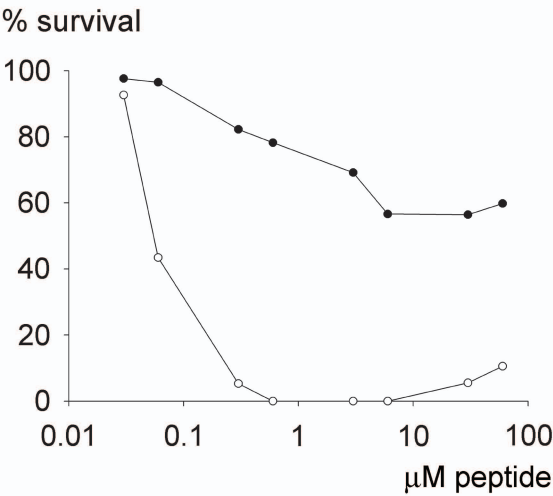
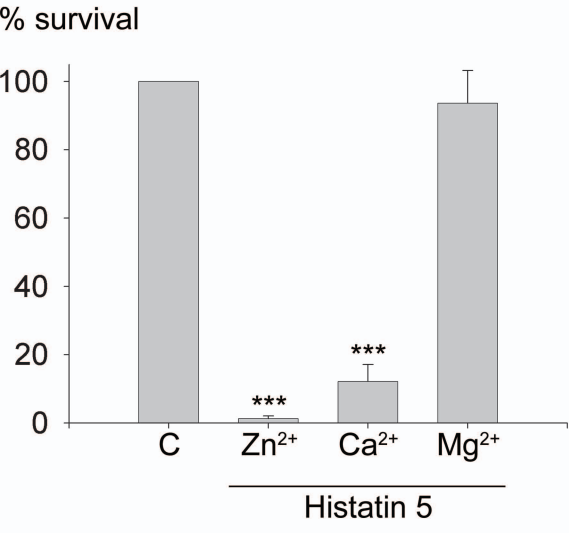

C
% survival
100
80
60
40
20
0
KHN20
100
80
60
40
20
0
GGH20
0.01
0.1
1
10
100
µM peptide

Figure 4

A


% survival
100
80
60
40
20
0
0.01
0.1
1
10
100
μM peptide

B


% survival
100
80
60
40
20
0
***
***
C
Zn2+
Ca2+
Mg2+
Histatin 5