Stability of antimicrobial activity of cryptdin-2 against selected pathogens under physiological conditions

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ABSTRACT

Background and Objective: An initial step prior to clinical development of any therapeutically active peptide is to evaluate its stability under physiological conditions. As cationic antimicrobial peptides have been reported to lose their activity under physiological conditions, present study was done to evaluate the stability of antimicrobial activity of cryptdin-2 (a Paneth cell antimicrobial peptide) against *Salmonella* Typhimurium, *Yersinia enterocolitica* and *Staphylococcus aureus* in the presence and absence of physiological concentrations of bile salts, monovalent and divalent cations, trypsin as well as at various pH values.

Methods: The antimicrobial activity of cryptdin-2 under various physiological conditions against *Salmonella* Typhimurium NCTC 74, *Yersinia enterocolitica* and *Staphylococcus aureus* was evaluated by use of a modified broth dilution technique

Results: Interestingly, the activity of the peptide against the Gram negative strains was augmented by bile salts while no change in the activity against *S. aureus* was observed. Though there was a decrease in activity with increasing concentrations of metal ions, the activity was not completely lost. The peptide was able to sustain its activity against all the three test strains at physiological concentrations of trypsin. At pH 8, no change in activity was observed against *Y. enterocolitica* and *S.* Typhimurium while it was found to be reduced against *S. aureus.*

Interpretation and Conclusion: The study provides data showing the stability of the peptide under the physiological conditions and indicates towards the possibility of developing it as an alternate strategy to combat bacterial pathogens.

Keywords: antimicrobial activity, bacterial pathogens, cryptdin-2, physiological conditions, stability.

INTRODUCTION

The emergence of bacterial resistance to common antibiotics poses a serious threat to human health and has rekindled interest in the development of novel therapeutic agents having broad antimicrobial mechanism viz. conventional antibiotics.[1] In this regard, cationic antimicrobial peptides (AMPs) also called defensins, of eukaryotic origin have gained considerable interest and are considered potential candidates as new therapeutic agents against bacterial infections. [2]

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AMPs are effector molecules of the innate immune system and possess direct antibacterial and immunomodulatory properties.[3] Earlier studies have indicated the *in vitro* and *in vivo* therapeutic potential of various cationic AMPs including human neutrophil peptides against bacterial[4] and viral infections.[5,6] The antibacterial activity of cryptdins (mouse Paneth cell defensins) has been investigated *in vitro* only against a few microbes.[7] Recently we have reported that cryptdin-2 displays a strong therapeutic activity against experimental Salmonellosis.[8] This prompted us to assess the stability and sustainability of this peptide under the conditions more closely related to those encountered *in vivo* (as an initial step of paramount importance) prior to its clinical development as a therapeutic agent. To the best of our knowledge, the stability of antimicrobial activity of cryptdin-2 against *S.* Typhimurium, *Y. enterocolitica* and *Staphylococcus aureus* under the conditions mimicking the *in-vivo* situations has been evaluated for the first time in this study.

MATERIALS AND METHODS

Bacterial strains and growth media

Salmonella Typhimurium NCTC 74, procured from Central Research Institute, Kasauli, India was used in the present study. This strain was maintained on MacConkey's agar medium and has been used in earlier studies both as a virulent as well as reference strain.[8-10] *Y. enterocolitica* ATCC 23715 and *Staphylococcus aureus* subsp. *aureus* ATCC 9144 were procured from Institute of Microbial Technology, Chandigarh, India and were maintained on Tryptose agar medium containing 0.005% thiamine HCl and nutrient agar medium respectively. To prepare the cell suspensions, the overnight cultures were harvested by centrifugation (3783 × g, 10 min), washed once with 10 mM sodium phosphate-buffered saline (PBS, pH 7.2) and resuspended in PBS. For killing assays, approx. 1×10^7 cfu/ml of *S.* Typhimurium and *S. aureus* were used while for *Y. enterocolitica*, the final cell count was adjusted to 1×10^9 cfu/ml using PBS (pH 7.4).

Synthetic cryptdin-2

Chemically synthesized peptide with an amino acid sequence of LRDLVCYCRTRGCKRRERMNGTC RKGHLMYTLCCR identical to the sequence of mouse Paneth cell cryptdin-2 with disulfide linkages between Cys-^{I}-Cys-^{VI}, Cys ^{II}-Cys ^{IV}, Cys^{III}-Cys^{V} was procured form Taurus Scientific, USA. It was suspended in 0.01% acetic acid, stored as a stock solution of 100mg/l at -20°C and was used within three weeks.

Minimum bactericidal concentrations (MBCs)

The antimicrobial activity of cryptdin-2 against *Salmonella Typhimurium* NCTC 74, *Yersinia enterocolitica* and *Staphylococcus aureus* was evaluated by use of a broth dilution technique. In brief, flat-bottom tubes containing bacterial cells in Mueller Hinton Broth and peptide at different concentrations were incubated at 37°C on an orbital shaker until the mid-exponential phase (16–18 h). The MBC (at which there was 99.99% inhibition of growth) was calculated by monitoring the colony forming units (CFUs) at various concentrations with respect to the untreated cells. The antibacterial effect was estimated as the rate of cells surviving against the total number of cells used. In addition, growth was monitored during the mid-log phase by measuring the optical density at 620 nm.

Minimum bactericidal concentrations (MBCs) in presence of bile salts

This was done by a similar method as described above with a slight modification. All the flat bottom flasks containing various concentrations of crytpdin-2 were supplemented with 0.3 % of sodium taurocholate and sodium deoxycholate. After the addition of the respective bacterial cells, these flasks were incubated under microaerophillic conditions in anaerobic jars. For determination of CFUs, 0.1ml of samples was plated on an appropriate agar plate for each species (MacConkey's agar for *Salmonella Typhimurium*, Tryptose soy broth (TSB) and Nutrient agar for *Y. enterocolitica* and *Staphylococcus aureus* respectively). The plates were then incubated at 37°C (under microaerophillic conditions) for 16-18h and used for colony counts measurements.

Effect of metal ions

The effect of metal ions on the antimicrobial activity was determined at MBCs of cryptdin-2 against the test bacterial strains. Mueller Hinton broth containing cryptdin-2 at its respective MBC against the individual bacteria was used in the assay as described above. Two monovalent cations (Na^+ and K^+) and two divalent cations (Ca^{2+} and Mg^{2+}) were used as chloride salts. NaCl or KCl was added to the media to final concentrations of 0, 10, 50, 100, 200 and 500 mM whereas $CaCl_2$ or $MgCl_2$ was added to the media to final concentrations of 0, 1, 2, 5, 10 and 20 mM. The percentage of bactericidal activity compared with that using 10mM phosphate buffer (pH 7.4) was calculated.

Effect of pH

The effect of pH on the antimicrobial activity of the cryptdin-2 was tested by determining their MBCs against the selected pathogens at different pH values. The pH of the media was altered by adding either 5 M HCl or NaOH. The antibacterial activity was tested at pH values ranging from pH 5 - 8.

Antimicrobial activity in presence of trypsin

Activity of cryptdin-2 against the selected pathogens was also studied in the presence and absence of trypsin following the method by Porter *et al.*[11] with a slight modification. Bacteria were suspended in 10mM sodium phosphate buffer (PB) (pH 7.4) containing 1% TSB supplemented or unsupplemented with 25µg/ml or 125µg/ml of trypsin and mixed with various concentrations of cryptdin-2 and incubated for 3 hours at 37°C. Then, the

CFUs were enumerated (in triplicates) on MacConkey's agar plates after making appropriate dilutions. An appropriate dilution of the reaction mixture in PB (100µl) was plated on an appropriate agar plate for each species (MacConkey's agar for *Salmonella* Typhimurium, TSB for *Yersinia enterocolitica* and Nutrient agar for *Staphylococcus aureus*) and then incubated at 37°C overnight. Inoculum density (CFU/ml) was calculated from the number of colonies on each plate. The antibacterial effect was estimated as the rate of cells surviving against the total number of cells used.

STATISTICAL ANALYSIS

To determine the significance level of the differences observed between the samples, one way analysis of variance (ANOVA) was performed followed by pair wise comparison procedures (Tukey test) using Jandel Sigma Stat Statistical Software, version 2.0. In all cases, statistical significance was defined as p value of < 0.05.

RESULTS

Minimum bactericidal concentrations (MBCs)

Cryptdin-2 decreased the colony forming units of all the test bacterial strains in a concentration dependent manner. When *Salmonella* Typhimurium, *Y. enteocolitica* and *S. aureus* cells were incubated with 5, 10, 15, 20 and 25 mg/l cryptdin-2, no visible growth was observed at concentrations 20 mg/l cryptdin-2 for *S.* Typhimuirum, 25 mg/l for *Y. enterocolitica* and 15 mg/l for *S. aureus*. To evaluate the MBC of cryptdin-2, bacterial cells were incubated with 15, 16, 17, 18, 19 or 20 mg/l cryptdin-2. No growth of *Salmonella* Typhimurium was observed when the cells were incubated with 19 mg/l and higher concentrations of cryptdin-2, indicating this concentration as the MBC (as reported earlier).[8] Similarly for *Y. enterocolitica*, bacterial cells were incubated with 20, 21, 22, 23, 24 or 25 mg/l cryptdin-2 and the MBC was evaluated as 24mg/l against *Y. enterocolitica*. While for *S. aureus*, the growth was completely inhibited at 15mg/l of cryptdin-2. Therefore, to evaluate the MBC, *S. aureus* cells were incubated with 10, 11, 12, 13, 14 or 15mg/l of cryptdin-2 and the MBC against *S. aureus* was determined to be 12mg/l (Fig.1).

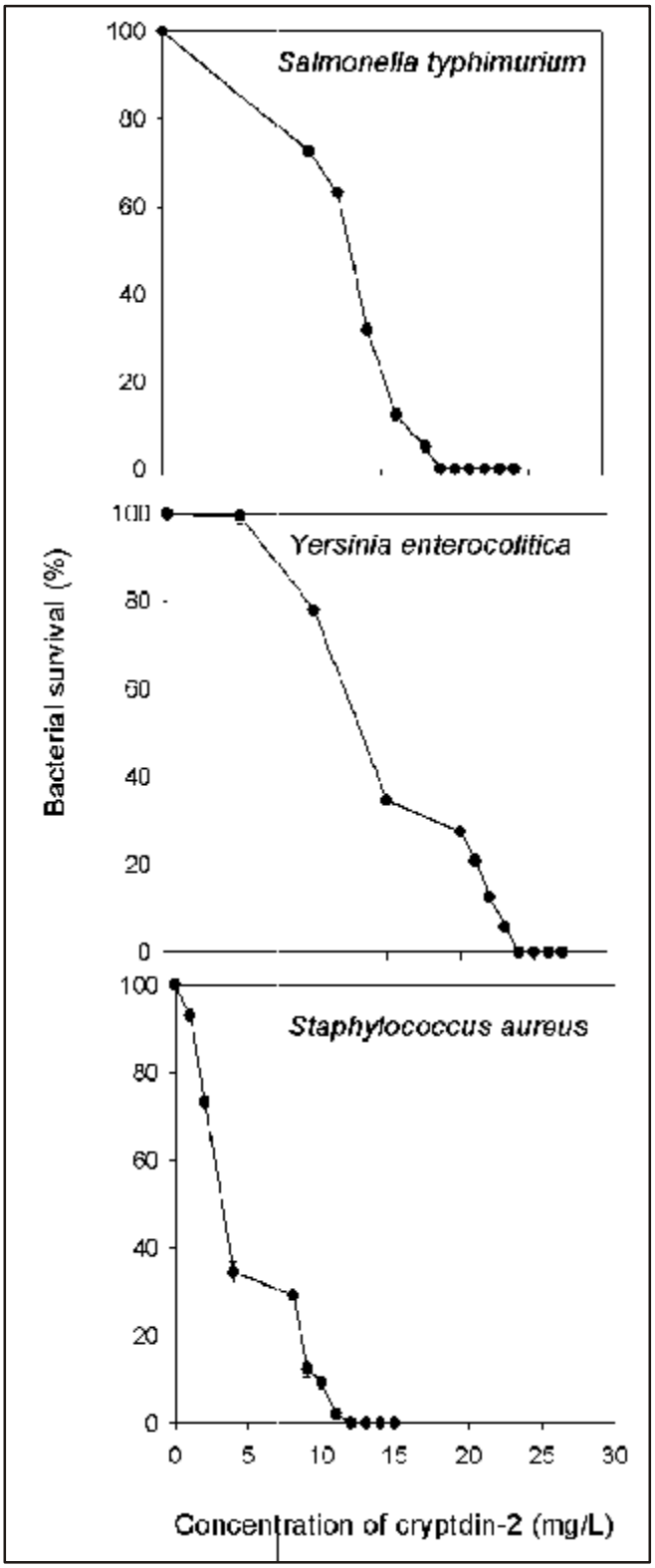

Figure-1. Antibacterial activity of cryptdin-2 against Yersinia enterocolitica, S. Typhimurium, and Staphylococcus aureus. Percentage of bacterial survival in the presence of cryptdin-2 compared with that without cryptdin-2 is represented on the longitudinal axis. The results represent the Mean ± SD from three independent experiments.

Effect of bile salts and microaerophilic conditions

Cryptdin-2 decreased the colony forming units of all the test bacterial strains in a concentration dependent manner in presence of bile salts also. However, the MBCs of cryptdin-2 against the pathogens were slightly altered in

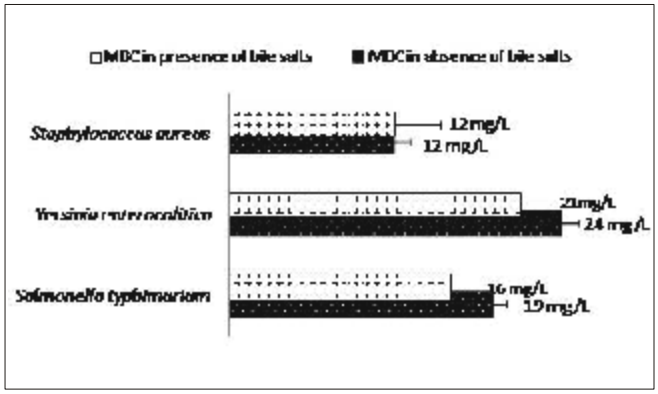

Figure-2. Minimum bactericidal concentrations of cryptdin-2 against the pathogens in presence and absence of 0.3% sodium taurocholate and sodium deoxycholate. The results represent the Mean ± SD from three independent experiments.

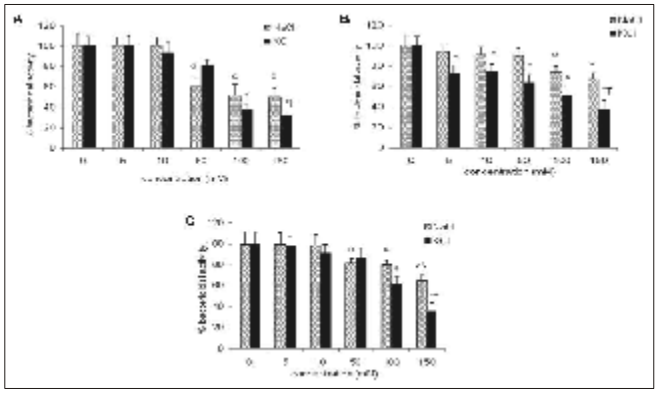

*Figure-3 A). Effect of NaCl and KCl on the bactericidal activity of cryptdin-2 against Y. enterocolitica. The results are means ± SD from three independent experiments. αp< 0.05 vs % age bactericidal activity against Y. enterocolitica at 0, 5, 10mM NaCl; *p<0.05 vs. %age bactericidal activity at 0, 5,10mM KCl; †p< 0.05 vs % age bactericidal activity at 50mM KCl; B). Effect of NaCl and KCl on bactericidal activity of cryptdin-2 against S. Typhimurium. αp< 0.05 vs. %age bactericidal activity against S. Typhimurium at 0,5,10 mM NaCl; *p< 0.05 vs. %age bactericidal activity in absence of KCl; †p< 0.05 vs. %age bactericidal activity at 50mM KCl; C). Effect of NaCl and KCl on bactericidal activity of cryptdin-2 against S. aureus. Values are means ± SD of three independent experiments. αp< 0.05 vs % age bactericidal activity against S. aureus at 0, 5mM NaCl; βp< 0.05 vs. %age bactericidal activity at 100mM NaCl; *p< 0.05 vs. %age bactericidal activity at 0, 5,10mM KCl; †p< 0.05 vs. %age bactericidal activity at 100mM KCl.*

presence of the bile salts. Interestingly, comparatively higher decrease in colony forming units at lower concentrations was observed for both *S.* Typhimurium and *Y. enterocolitica* when bile salts were added to the test medium in the presence of cryptdin-2. The MBC values in presence of bile salts were evaluated to be 16mg/l and 21mg/l for *Salmonella* and *Yersinia* respectively. While for *S. aureus*, no change in MBC value of cryptdin-2 in presence of bile salts was observed (Fig. 2).

Effect of monovalent cations

When the effect of monovalent cations on the antibacterial activity of cryptdin-2 was evaluated at its respective minimum bactericidal concentrations against each organism, a loss in activity was observed at higher concentrations of these ions. At 5mM NaCl, no significant reduction ($p > 0.05$) in the antimicrobial activity of cryptdin-2 was observed against all the three pathogens. In the presence of 150mM NaCl concentration, 50% reduction ($p<0.05$) in activity against *Y. enterocolitica* was observed (Fig.3A). However, the antibacterial activity against *S.* Typhimurium (Fig. 3B) and *S. aureus* (Fig.3C) showed 32% ($p< 0.05$) and 36% reduction ($p<0.05$) respectively at 150mM NaCl. On the other hand, 5mM KCl showed a higher influence on the bactericidal activity against *S.* Typhimurium (Fig.3B) than *S. aureus* (Fig.3C) and *Y. enterocolitica* (Fig.3A) reducing the antibacterial activity to 72.8% while for *Staphylococcus* and *Yersinia*, the activity was still 99.99% and 97.8% at 5mM KCl. However, at higher 150mM KCl concentration, comparatively higher reduction in activity against *Y. enterocolitica* (69% reduction) ($p< 0.05$) and *S. aureus* (64% reduction) ($p< 0.05$) was observed as compared to *S.* Typhimurium (62% reduction) ($p< 0.05$).

Effect of Divalent Cations

Cryptdin-2 was able to retain its antimicrobial activity against all the selected strains even upto 20mM Ca^{2+} and Mg^{2+} ions. However, with increasing concentrations of these cations, the antibacterial activity was found to be decreased. The presence of divalent cations in the test medium had a higher influence on the antibacterial activity of cryptdin-2 than the monovalent cations. At 1mM $MgCl_2$ concentration, no significant change ($p>0.05$) in antibacterial activity against *Y. enterocolitica* (Fig.4A) and *S.* Typhimurium (Fig.4B) was observed, whereas the activity was reduced to 91% ($p<0.05$) against *S. aureus* (Fig.4C). Approx. 80% antibacterial activity was still retained against all the pathogens at about 2mM $MgCl_2$. However, at 20mM $MgCl_2$, maximum reduction in antibacterial activity was observed against *Y. enterocolitica* (65.5% reduction) followed by *S. aureus* (58% reduction). Least effect of 20mM MgCl2 was observed against *S.* Typhimurium where 38% reduction in the antibacterial activity was there. Supplementation of the test medium with $CaCl_2$ affected the antibacterial activity to a larger extent as compared to $MgCl_2$. At 1mM

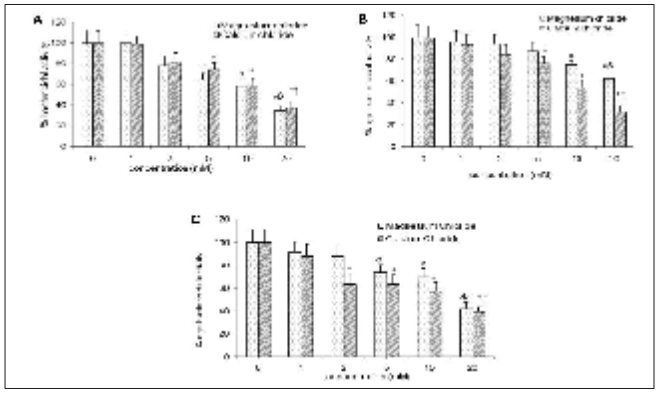

*Figure-4 A). Effect of MgCl2 and CaCl2 on antimicrobial activity of cryptdin-2 against Y.enterocolitica.αp< 0.05 vs. %age bactericidal activity at 0, 1, 2mM MgCl2; βp< 0.05 vs. %age bactericidal activity at 10mM MgCl2; *p<0.05 vs %age bactericidal activity at 0,1mM CaCl2; †p<0.05 vs. %age bactericidal activity at 10mM CaCl2; B) .Effect of MgCl2 and CaCl2 on antimicrobial activity of cryptdin-2 against S. Typhimurium. αp< 0.05 vs. %age bactericidal activity at 0, 1, 2mM MgCl2; βp< 0.05 vs. %age bactericidal activity at 10mM MgCl2; *p<0.05 vs. %age bactericidal activity in the absence of CaCl2; †p<0.05 vs. %age bactericidal activity at 10mM CaCl2; C). Effect of MgCl2 and CaCl2 on antimicrobial activity of cryptdin-2 against S. aureus. αp<0.05 vs. %age bactericidal activity in the absence of MgCl2; βp<0.05 vs. %age bactericidal activity at 10mM MgCl2; *p<0.05 vs. %age bactericidal activity at 0,1 mM CaCl2; †p<0.05 vs. %age bactericidal activity at 10mM CaCl2.*

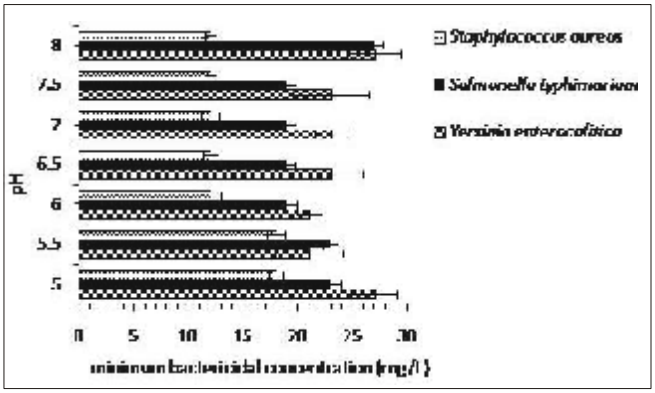

Figure-5. Minimum bactericidal concentrations of cryptdin-2 against the pathogens at various pH values. Values are means ± SD of three independent experiments.

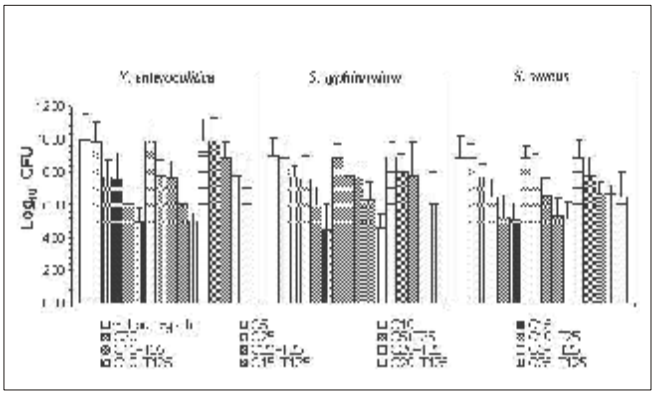

Figure-6 Log10 decrease in CFU of the test pathogens by cryptdin-2 at various concentrations in presence (25µg/ml, T25; 125µg/ml, T125 of trypsin after 3h. Values are means ± SD of three independent experiments.

$CaCl_2$, the reduction in activity against *S.* Typhimurium (Fig.4B), *Y. enterocolitica* (Fig.4A) and *S. aureus* (Fig. 4C) was 7% (p>0.05), 2% (p>0.05) and 12% (p<0.05) respectively. At 2mM $CaCl_2$, greater than 80% activity was retained against *S.* Typhimurium and *Y. enterocolitica* but against *S. aureus* the activity was reduced to 63.14%. However, at 20mM $CaCl_2$, the reduction was higher (p<0.05) i.e 63%, 68% and 60% against *Y. enterocolitica*, *S.* Typhimurium and *S. aureus*, respectively. The growth of bacteria in all the flasks in the absence of the peptide suggested that there was no influence of the salt on bacterial growth

Effect of pH

The effect of pH on the activity of the peptide was established by determining the MBC of each of the peptide against all the three selected strains at pH values varying from 5 to 8 (Fig.5).The MBC was not affected against *Salmonella* Typhimurium between the pH range of 6 to 7.5. Although the MIC of the peptide against *S. aureus* was markedly affected below 6.5 pH, but it did not change significantly against *Yersinia enterocolitica* between the pH range 5.5 to 7.5.

Antimicrobial activity in presence of trypsin

The antimicrobial activity of cryptdin-2 against the bacterial strains was tested against the test pathogens in presence of 25 and 125 µg/ml of trypsin. No significant change (p>0.05) in the log unit decrease was observed when the bacterial cells were incubated with various concentrations of cryptdin-2 in the presence or absence of 25µg/ml of trypsin indicating the relative stability of the peptide to trypsin degradation. The decrease in CFUs in presence of cryptdin-2 in a concentration dependent manner was still observed in the presence of trypsin (125µg/ml) giving appx. 2.4 log unit decrease in CFU of *S. aureus* and *Y. enterocolitica* while a 2.83 log unit decrease was observed against *S.* Typhimurium (Fig.6)

DISCUSSION

The therapeutic success of an agent against oral or systemic infections is relative to its stability under the physiological conditions. Keeping this in view, the present study was carried out to assess the effect of various

physiological parameters prevailing inside the dynamic host-milieu on the antimicrobial activity of cryptdin-2 against three selected pathogens i.e *Salmonella* Typhimurium, *Y. enterocolitica* and *Staphylococcus aureus*. The peptide was found to be active against both Gram positive and Gram negative bacteria akin to other mammalian defensins.[12, 13] Comparatively, cryptdin-2 was found to be more effective against *S. aureus* than the Gram negative pathogens in concordance with our earlier report.[14] This difference in susceptibility might be due to the nature of interactions between antimicrobial peptides and microbial membrane components present in Gram positive and Gram negative bacteria. Similar variation in potency against various bacterial strains has been observed earlier for murine cryptdins.[15]

The antibacterial activity of cryptdin-2 against the pathogens in terms of MBC in the presence of bile salts at physiological concentrations was also evaluated. The MBCs against *S.* Typhimurium and *Y. enterocolitica* were found to be lower when the assay medium was supplemented with 0.3%w/v concentrations of sodium taurocholate and sodium deoxycholate. The increased antimicrobial effect with addition of bile salts may be related to the increased solubility of the peptide in presence of bile salts.[16] Additionally, bile salts may also alter the structural integrity of bacterial membrane thereby enhancing the uptake of the peptide. However, no change was observed in MBC against *S. aureus* in the presence of bile salts. It might be attributed to the thick peptidoglycan layer that might be a barrier in access of bile salts to the cell membrane.

A major obstacle in the development of AMPs as novel antibiotics is the antagonism between the peptides and ionic strength (in their environment), thereby impairing the practical therapeutic use of these peptides.[17] Many cationic antimicrobial peptides including HD-5,[18,19] lactoferricin B,[20] histain 5,[21] human cathelicidin LL-37,[22] protegrins[23] and pleurocidin,[24] have been reported to be salt sensitive and have been shown to lose their antimicrobial activity at elevated concentrations of mono- or divalent cations. In this study, the activity of cryptdin-2 evaluated in presence of physiological concentrations of monovalent and divalent cations was found to be influenced by elevated concentrations of both monovalent and divalent cations. It has been suggested that the presence of cations can prevent the peptides from interacting with the membrane and subsequently neutralize the capability of peptides to kill bacteria.[25]

As the pH in small intestine ranging from pH 5 to 8 (from proximal to distal end)[26-28] can affect the activity of cryptdin-2 (by modifying the peptide or the target), the influence of pH changes on the activity was also monitored. The activity against all the three pathogens decreased at pH 5.5. It has been reported that resistance of pathogens to intestinal defensins is increased due to the upregulation of acid tolerance response systems under the acidic stress conditions as has been suggested for *Salmoenlla* Typhimurium.[11] However, at pH 8, no change in activity was observed against *Y. enterocolitica* and *S.* Typhimurium while it was found to be reduced against *S. aureus.* This might be attributed to the fact that properties of the bacterial targets are differentially modified by the pH changes.[29-31] In addition, the net charge of cryptdin-2 which parallels its antimicrobial activity could also be influenced by the pH leading to alterations in its antibacterial activity.[12, 32, 33]

As cryptdin-2 might encounter many proteases in the luminal fluids, its stability to trypsin degradation was also evaluated in this study. Surprisingly, the peptide was able to sustain its activity against all the three test strains at physiological concentrations of trypsin. Although the activity was lesser at 125µg/ml of trypsin, a 2 log unit decrease was still observed in colony forming units of all the test strains. Our observations are in concordance with earlier studies which suggested that the rigid cysteine bridging motif of defensins possibly blocks the access of trypsin to susceptible sites.[11] Moreover, *in-vivo* intracellular and secreted intestinal defensins may be further protected by trypsin inhibitors, which are also reported to be present in Paneth cells.

The persistence of the antimicrobial activity of cryptdin-2 against the pathogens under various conditions simulating the host *in-vivo* milieu indicates towards its possible clinical development as an effective strategy to combat bacterial infections. Effectiveness of the peptide through increased stability and protection from inactivation (particularly in reference to cations and pH) may further be increased by encapsulation methods.[34] These methods would also allow sustained diffusion of the active peptide and deliver a controlled dose over an extended period of time.

ACKNOWLEDGEMENTS

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Conflict of interest

The authors declare that they have no conflict of interest

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