Structure-activity relationship of buffalo antibacterial hepcidin analogs

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Hepcidin is an anti-microbial peptide expressed predominantly in the liver of many species. Based on the amino acid sequence deduced from buffalo (Bubalus bubalis) hepcidin cDNA (Accession no. EU399814), six peptides Hepc₁-25, Hepc₆-25, Hepc₁₁-25, Hepc₁₅-2₅, and Hepc₁₅-2₅ were synthesized using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry. CD spectroscopy revealed different spectra of the peptides in different solvents and in all the cases β-structure was found to be dominant with less α-helix as predicted. Quantitation of secondary structure indicated the highest β-structure for all the six peptides in SDS solution, when used as mimetic for membrane-like environment. The CD spectra of all the peptides taken in water showed that degree of randomness decreased with increase in chain length of the peptide. Out of the six peptides, only Hepc₁-2₅, Hepc₆-2₅ and Hepc₁₁-2₅ showed antibacterial activity against Staphylococcus aureus (Gram-positive bacteria). The peptides did not show any sensitivity toward E. coli (Gram-negative bacteria). Minimum inhibitory concentration (MIC) showed the lowest value for Hepc₁-2₅ as an antibacterial agent, followed by Hepc₆-2₅ and Hepc₁₁-2₅. The peptides Hepc₁₅-2₅ and Hepc₁₅-2₅ with more random structure did not show any antimicrobial activity. The study demonstrated that 5 amino acids at N-terminal in buffalo hepcidin can be truncated without loss of antimicrobial activity and further reduction of length of the analog from 20 to 19 amino acids resulted increase in the activity because of increase in β-structure of the peptide shown by CD spectroscopy.

Keywords: Anti-microbial peptide, Circular dichroism spectroscopy, β-Structure, Minimum inhibitory concentration, Hepcidin analogs, Staphylococcus aureus, E. coli

Anti-microbial peptides (AMPs) are a broadly distributed group of molecules that are important in host defense against microbial invasion.¹³ The largest group of antimicrobial peptides is that of cationic antimicrobial peptides that are found in both cationic and hydrophobic surfaces enabling them to insert into biological membranes.⁴ Cationic peptides are divided into three classes on the basis of their structural features: (i) linear peptides forming α-helical structures e.g. cecropins, (ii) cysteine-rich open-ended peptides containing single or several disulfide bridges e.g. defensins (α, β and θ-defensins)⁶ and hepcidin⁷, and (iii) molecules rich in specific amino acids, such as proline, glycine or histidine e.g. indolicidin. The other classes of antimicrobial peptides are anionic peptides, aromatic dipeptides and peptides derived from oxygen-binding proteins. The antimicrobial effect of AMPs involve the direct electrostatic interaction with negatively charged microbial cytoplasmic membrane, followed by physical disruption and killing of a broad spectrum of microorganisms due to lack of involvement of specific receptors.⁸ The fundamental differences that exist between microbial and mammalian cells represent targets for antimicrobial peptides. Many antimicrobial peptides employ sophisticated and dynamic mechanisms of action to effect rapid and potent activities consistent with their likely roles in antimicrobial host defense.⁹

Bacteria differ in their intrinsic susceptibility to AMPs and relative resistance to some of these is now well recognised.¹⁰⁻¹² However, AMPs possess a broad spectrum of potential antibacterial activity. These peptides kill microorganisms rapidly compared to other antibiotics and appear to be alternate to the development of resistance. Hepcidin (Hepc) is a novel gene-encoded, 25-residues, 2-3 kDa, cysteine-rich and hepcidin cDNA (Accession no. EU399814), six peptides Hepc₁-2₅, Hepc₆-2₅, Hepc₁₁-2₅ and Hepc₁₅-2₅ were synthesized using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry. CD spectroscopy revealed different spectra of the peptides in different solvents and in all the cases β-structure was found to be dominant with less α-helix as predicted. Quantitation of secondary structure indicated the highest β-structure for all the six peptides in SDS solution, when used as mimetic for membrane-like environment. The CD spectra of all the peptides taken in water showed that degree of randomness decreased with increase in chain length of the peptide. Out of the six peptides, only Hepc₁-2₅, Hepc₆-2₅ and Hepc₁₁-2₅ showed antibacterial activity against Staphylococcus aureus (Gram-positive bacteria). The peptides did not show any sensitivity toward E. coli (Gram-negative bacteria). Minimum inhibitory concentration (MIC) showed the lowest value for Hepc₁-2₅ as an antibacterial agent, followed by Hepc₆-2₅ and Hepc₁₁-2₅. The peptides Hepc₁₅-2₅, Hepc₁₅-2₅ and Hepc₁₅-2₅ with more random structure did not show any antimicrobial activity. The study demonstrated that 5 amino acids at N-terminal in buffalo hepcidin can be truncated without loss of antimicrobial activity and further reduction of length of the analog from 20 to 19 amino acids resulted increase in the activity because of increase in β-structure of the peptide shown by CD spectroscopy.

Keywords: Anti-microbial peptide, Circular dichroism spectroscopy, β-Structure, Minimum inhibitory concentration, Hepcidin analogs, Staphylococcus aureus, E. coli
Buffalo (*Bubalus bubalis*) is well known to resist naturally against microbial infection in better way and may provide insight into natural safeguard acquired through this defensin-like peptide hepcidin. In this report, various hepcidin analogs designed on the basis of deduced amino acids sequence of *B. bubalis* hepcidin have been synthesized using solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry and their structure-activity relationship with respect to their antibacterial activity against *S. aureus* and *E. coli* has been studied.

**Materials and Methods**

**Peptide designing and synthesis**

Six different buffalo hepcidin analogs (Table 1) were designed on the basis of derived amino acid sequences of *Bubalus bubalis* hepcidin (Accession no. EU399814). Hepc<sub>1-25</sub> (25 residues) is the full length buffalo hepcidin, while other N-terminal truncated forms Hepc<sub>6-25</sub> (20 residues) and Hepc<sub>7-25</sub> (19 residues) have eight cysteine residues engaged in four disulfide bonds and Hepc<sub>9-25</sub> (17 residues), Hepc<sub>12-25</sub> (14 residues) and Hepc<sub>15-25</sub> (11 residues) have reduced number of cysteine residues. The peptides were synthesized by solid-phase Fmoc chemistry. Each N-terminal and side chain-protected amino acid was coupled to the Wang resin as amino acid ester formed after activation by 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxy benzotriazole (HOBt).

The resin-bound peptides were de-protected and cleaved from resin support by treating them with cleavage mixture comprising trifluoroacetic acid (TFA): thioanisole:water (95:2.5:2.5,v/v). Cleavage mixture was added at 200 µl/tube and left at room temperature for 5 h with continuous shaking at moderate speed. The tubes were centrifuged at 14,000 g (Heraeus Biofuge, Fresco) for 5 min and the supernatant was transferred carefully to different tubes and evaporated under vacuum. Peptides were precipitated by adding chilled dry ether. The peptide analogs were purified by high performance liquid chromatography (HPLC, Shimadzu Class VP) using C18 column, equilibrated with 0.1% TFA in water. After injecting the sample, desalting was done with 10% acetonitrile in 0.1% TFA. The peptide peak was eluted with 40% acetonitrile in 0.1% TFA at a flow rate of 0.3 ml per min and absorbance was recorded at 220 nm.

**Circular dichroism spectroscopy**

Circular dichroism (CD) spectroscopy of synthesized peptide analogues was carried out on JASCO J-810 Spectropolarimeter (Jasco Corp., Tokyo, Japan) in the far ultra-violet (UV) range (190-260 nm) using quartz cuvettes of 0.1 cm path length and 1 nm bandwidth at 0.1 nm resolution. Each spectrum was recorded as an average of three repeated scans in a continuous scanning mode with 100 nm/s scanning speed with response time of 1 s. The contribution of buffer/solvent was subtracted from each spectrum. Molar ellipticity was calculated using 100 µg/ml of the peptide.

CD spectra of the peptides were recorded in different solvents, such as pure HPLC grade water, 10 mM sodium phosphate buffer (pH 7.4), trifluoroethanol (TFE, 100% and 50% v/v in water), 10% acetonitrile in 0.1% TFA. The peptide peak was eluted with 40% acetonitrile in 0.1% TFA. The peptide peak was eluted with 40% acetonitrile in 0.1% TFA at a flow rate of 0.3 ml per min and absorbance was recorded at 220 nm.

**Antimicrobial activity assay**

The antimicrobial activity of synthesized peptides was tested qualitatively against *S. aureus* (ATCC 6538) and *E. coli* (VT-3) by disc diffusion test using Muller Hilton agar. The minimum inhibitory concentration (MIC) of the peptides was determined on Luria-Bertani (LB) agar plate according to Amsterdam method. The organisms were grown in nutrient broth to mid-logarithmic phase and diluted to 10<sup>6</sup> colony forming unit (CFU)/ml in 10 mM sodium phosphate buffer (pH 7.0). Serial dilutions of the peptide analogs made in microtitre plates using LB broth; each was inoculated with 10 µl of test organism and incubated for 2 h at 37°C. The solvent of respective peptide analog was used as control. A loopfull of peptide-treated *S. aureus* culture from microtitre plate was streaked on LB plates having

**Table 1—Analogs of *Bubalus bubalis* hepcidin**

<table>
<thead>
<tr>
<th>Region</th>
<th>Amino acid sequences for peptide synthesis</th>
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<tbody>
<tr>
<td>Hepc&lt;sub&gt;1-25&lt;/sub&gt;</td>
<td>RKGTCGMC CRT</td>
</tr>
<tr>
<td>Hepc&lt;sub&gt;6-25&lt;/sub&gt;</td>
<td>GCCRKGTCGMC CRT</td>
</tr>
<tr>
<td>Hepc&lt;sub&gt;7-25&lt;/sub&gt;</td>
<td>FCCGRCRKGTGMCCRT</td>
</tr>
<tr>
<td>Hepc&lt;sub&gt;9-25&lt;/sub&gt;</td>
<td>CIFCCGRCRKGTGMCCRT</td>
</tr>
<tr>
<td>Hepc&lt;sub&gt;12-25&lt;/sub&gt;</td>
<td>ICIFCCGRCRKGTGMCCRT</td>
</tr>
<tr>
<td>Hepc&lt;sub&gt;15-25&lt;/sub&gt;</td>
<td>DTHFPICIFCCGRCRKGTGMCCRT</td>
</tr>
</tbody>
</table>
1.5% agarose and incubated for 18 h at 37°C. The lowest concentration preventing visible microbial growth was taken as MIC for the respective peptides.

**Cytotoxicity assay**
Relative toxicity of the peptides on eukaryotic cells (lymphocytes) was studied by measuring the permeability to propidium iodide (PI) by fluorescence activated cell sorter (FACS). Suspension of 10⁶ lymphocytes in 200 µl of PBS was incubated with different concentrations of peptides (100 and 200 µM) for 3 h and relative toxicity was analyzed employing cellquestpro software by measuring the permeability to PI.

**Results**

**Biophysical characterization of synthesized hepcidin analogs**
Predicted charge at pH 7.0 and mass of the synthesized buffalo hepcidin, Hepc₁-25, Hepc₆-25, Hepc₇-25, Hepc₉-25, Hepc₁₂-25 and Hepc₁₅-25 are presented in Table 2. CD spectra of the peptides in water at concentration of 0.1 and 0.3 mg/ml are presented in Fig. 1a and b respectively. Change in concentration of the peptides from 0.1 to 0.3 mg/ml affected the CD spectra accompanied by decrease in negative ellipticity. CD spectra of Hepc₁₅-25 in water had a negative trough at 198 nm which decreased to almost half with the addition of SDS. CD spectra of Hepc₁₂-25 in water had a negative trough at 220 nm with the addition of TFE resulted in appearance of negative CD band at around 200 nm in addition to 220 nm negative extrema present in CD spectra of peptide in water. CD spectra of Hepc₀-25 in water showed negative broad minimum around 220 nm. Addition of TFE led to appearance of a negative trough around 200-205 nm with another negative minimum at 220 nm. A more pronounced 220 nm negative band was observed in the CD spectra of the peptide in 20 mM SDS. CD spectra of the longer peptide Hepc₇-25 in water had a negative trough at 220 nm with a positive ellipticity at the region 192-200 nm. CD spectra had a cross-over to have positive ellipticity at 198 nm at θ (molar ellipticity) of +11000 degcm⁻²decimol⁻¹ and peptide in 100% TFE had a negative CD band at around 225 nm (Fig. 2). Addition of 20 mM SDS showed no change in CD band at 220 nm, but a prominent negative CD band with ellipticity of −5500 appeared at 202 nm. Hepc₀-25 as compared to Hepc₁₅-25 showed an increase in the positive ellipticity in CD at the region 190-200 nm with the appearance of positive CD band at 198 nm (molar ellipticity = 3800 degcm⁻²decimol⁻¹). Addition of TFE resulted in appearance of negative CD band around 200 nm in addition to 220 nm negative extrema present in CD spectra of peptide in water. CD spectra of Hepc₁₂-25 in water showed negative broad minimum around 220 nm. Addition of TFE led to appearance of a negative trough around 200-205 nm with another negative minimum at 220 nm.

![Fig. 1—CD Spectra of the peptides in water at concentration of 0.1 mg/ml (a) and 0.3 mg/ml (b) [1, Hepc₁₅-25, 2, Hepc₁₂-25, 3, Hepc₉-25, 4, Hepc₇-25, 5, Hepc₆-25, 6, Hepc₁-25]]}
spectra taken in SDS (20 mM) were also associated with isodichroictic point at 214 nm (Fig. 3).

CD spectra of full-length peptide Hepc\(_{1-25}\) in water had a strong positive CD band at around 200 nm (molar ellipticity = +8500 deg cm\(^2\) decimol\(^{-1}\)). Addition of TFE again resulted in appearance of a negative CD band at around 205 nm. In SDS (20 mM), the 198 nm positive band diminished and negative ellipticity in the region of 200-230 nm was also decreased (Fig. 4).

Quantitative estimation of different secondary structures made from observed CD spectra of different peptides are given in Table 3.

Microbicidal characterization of synthesized hepcidin analogs

Three synthesized peptides Hepc\(_{7-25}\), Hepc\(_{6-25}\) and Hepc\(_{1-25}\) out of six showed anti-bacterial activity against \textit{S. aureus} (Gram-positive), but not against \textit{E. coli} (Gram-negative). The other three shorter N-terminal truncated peptides Hepc\(_{9-25}\) (17 residues), Hepc\(_{12-25}\) (14 amino acids) and Hepc\(_{15-25}\) (11 amino acids) did not show any antibacterial activity against both the organisms. The antibacterial activity of all the three active peptides increased linearly with increase in concentration of the peptide. The minimum inhibitory concentration (MIC) of Hepc\(_{7-25}\), Hepc\(_{6-25}\) and Hepc\(_{1-25}\) against \textit{S. aureus} was 18, 23 and 25 µM, respectively. The three active peptides showed different spectra in terms of MIC on the tested organisms and MIC was found to be lowest for Hepc\(_{7-25}\), followed by Hepc\(_{6-25}\) and Hepc\(_{1-25}\).

Analysis of cytotoxicity by FACS

Cytotoxic effect of peptides on buffalo lymphocytes was determined at two different concentrations of the peptides by studying permeability to PI. The percent of death cells observed in control sample treated only with the solvent was 18.64. Damage to lymphocytes was not significant at 100 µM. At 200 µM, mild cytotoxicity was induced by the peptides and the highest percentage of dead cells was found to be 30.81 in Hepc\(_{6-25}\)-treated cells. The percent of permeabilized cells is presented in Table 4.
Ordered conformation is perhaps required for a peptide to produce its biological activity. This is supported by the earlier reports\(^9,23\) who have suggested that many of the antimicrobial peptides are unstructured in water, but adopt amphipathic structures upon interaction with the biological membranes or membrane-mimicking environments. In the present study, the percent of turn in the peptides when taken in water was much lesser than the predicted values and reduced further when the peptide concentration was increased from 0.1 to 0.3 mg/ml. The observation indicated that cysteine residues which form intramolecular disulfide bonds in monomeric form get involved in intermolecular disulfide bond in the presence of other hepcidin peptides in the close vicinity.

The antibacterial activity of the three biologically active peptides Hepc\(_{1-25}\), Hepc\(_{6-25}\) and Hepc\(_{7-25}\) against *S. aureus* and *E. coli* was in agreement with the observations of earlier study\(^13\), wherein it has been reported that gram-negative bacteria do not show sensitivity towards the synthetic peptide. The absence of antibacterial activity in the other three peptides Hepc\(_{9-25}\), Hepc\(_{12-25}\) and Hepc\(_{15-25}\) was possibly due to their random structure. Among the active peptides, Hepc\(_{7-25}\) was found to be most active as an antibacterial agent, followed by Hepc\(_{6-25}\) and Hepc\(_{1-25}\). Earlier, it is reported that the truncated form of hepcidin of 20 residues is more active than 25 residue hepcidin as bactericidal agent\(^14\). The lesser activity of Hepc\(_{1-25}\) than Hepc\(_{6-25}\) might be due the tendency of Hepc\(_{1-25}\) to aggregate readily, as compared to its truncated form which remains in monomeric form in solution\(^21\).

Anti-microbial nature of Hepcidin (25 and 20 residues) has been reported earlier and in this study, Hepc\(_{7-25}\) (19 amino acids) was also found to be antibacterial. Indeed, this 19 amino acid form showed a larger zone of inhibition, as compared to that of 20 and 25 amino acids form. It was in contrast with the findings of Nemeth and coworkers\(^20\), who opined that there is complete loss of activity, when five residues are removed from N-terminal side of active hepcidin. The MIC of the active peptides as determined by Amsterdam method\(^25\) revealed that it was lowest for Hepc\(_{7-25}\). The values were higher than 14 \(\mu\)M of human hepcidin\(^13\), but were within the range of bass hepcidin (5.5 to 44 \(\mu\)M)\(^24\).

The percentage of dead cells observed at control and the peptide-treated cells were within a very

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**Table 4**—Percentage of permeabilized cells following *in vitro* treatment with synthesized biological active hepcidin analogs

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Percentage of permeabilized cells at different peptide concentration</th>
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<tbody>
<tr>
<td></td>
<td>100 (\mu)M</td>
</tr>
<tr>
<td>Hepc (_{7-25})</td>
<td>23.49</td>
</tr>
<tr>
<td>Hepc (_{6-25})</td>
<td>24.28</td>
</tr>
<tr>
<td>Hepc (_{1-25})</td>
<td>23.16</td>
</tr>
</tbody>
</table>

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**Discussion**

The CD spectra of different peptides revealed the predominant \(\beta\)-conformation with less \(\alpha\)-helical propensity in different solutions and it was in close agreement with secondary structure predicted by Chou and Fasman method\(^17\) in protean of Lasergene (DNASStar Inc. USA). The degree of randomness decreased with increase in chain length which reflects that there is possibility of the longer peptide to be more active. This observation was in agreement with the finding of Nemeth and coworkers\(^20\) who have determined the ferroportin-binding activity of hepcidin in a cell line expressing ferroportin-green fluorescent protein (GFP) fusion protein by measuring the degradation of ferroportin-GFP and the accumulation of ferritin after the peptide treatment. In their study, they found that serial deletion of N-terminal amino acids cause progressive decrease in the activity. The metal-binding ability of hepcidin is also reported to decrease with progressive reduction of N-terminal amino acid and there is complete loss of such effect on removal of five amino-terminal amino acids\(^21\).

The peptides were predicted to have a high percent of turn which might be due to the presence of 8 cysteine residues within a stretch of 17 amino acids. It has been reported that hepcidin peptide when folded correctly gives a constrained bent structure because of the intramolecular disulfide bonds\(^22\).

Quantitative analysis of secondary structure of the spectra showed predominantly \(\beta\)-structure and the highest percent of \(\beta\)-structure was observed in 20 mM SDS. This concentration of SDS is above its critical concentration of micelle formation which can provide prokaryotic membrane-like environment to the peptides and thus the conformation can be considered as the interaction with the bacterial cell membrane. The predominant \(\beta\)-structure of all the peptides in 20 mM SDS (which mimics the prokaryotic cell membrane) in the present study also indicated that the peptides remained in its most ordered form when in contact with the bacterial cell membrane.
narrow range, as revealed by the determination of cell cytotoxicity by FACS. This high percentage of cell death (18.64%) in the control might be because of the TFE present in the vehicle. The percent permeabilized cells treated with 100 µM of Hepc7-25, Hepc6-25 and Hepc1-25 was 23.49, 24.28 and 23.16, respectively after 3 h incubation which was insignificant as compared to control and the cytotoxicity at peptide concentration of 200 µM can be considered as mild. The insignificant cytotoxicity was in consistent with the earlier finding 14, wherein it is reported that hepcidin peptide is far less cytotoxic than α-defensin and is not cytotoxic at 3000-fold higher concentration than that found in the urine.

Conclusion
The chemically synthesized buffalo hepcidin analogs revealed predominant β-structure in CD spectroscopy, which was required by a peptide to produce its antibacterial activity. The study demonstrated that the longer peptides Hepc1-25, Hepc6-25 and Hepc7-25 with more ordered structure exhibited antibacterial activity against S. aureus. Cytotoxic concentrations of the active peptides were above 4-fold of MIC and hence it can be concluded that hepcidin, Hepc1-25 and its truncated forms Hepc6-25 and Hepc7-25 can serve as template for designing of new molecules of drugs.

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References