3.1 Introduction

Disulfide bridges between cysteine residues are the key structural elements of many secretory proteins, peptides, enzymes, immunoglobulins and inhibitors (Thornton, 1981; Creighton, 1988; Betz, 1991). Motifs containing repeated clusters of cysteine residues also appear in the extracellular domains of membrane bound receptors, although they do not form disulfide bridges (Chamberlain and Burgoyne, 1998; Chamberlain and Burgoyne, 2000). Introduction of disulfide bridges into natural or designed peptides has been carried out with the goal to improve biological activities (Harwig et al., 1996; Krishnakumari et al., 1999; Zhang et al., 2000; Yu et al., 2000).

Chemical synthesis of multiple disulfide containing peptides may not result in a homogeneous population containing a particular disulfide linkage. This is mainly because a molecule containing 2N cysteine residues
can be cross linked intramolecularly in \((2N)! / (2^N \cdot N!)\) different ways. Thus, a peptide with six cysteines upon oxidation could theoretically yield 15 different possible disulfide cross linking patterns. Similarly, a peptide with four cysteines can crosslink in 3 different ways.

Defensins are composed of 30-40 amino acid residues, including 6 invariant, highly conserved cysteines, which cyclises in a particular pattern to render it a complexly folded, triple stranded antiparallel \(\beta\)-sheet structure. Depending on the position and linkages of the six cysteines, vertebrate defensins are classified into \(\alpha\)-, \(\beta\)- and recently reported \(\theta\)-defensins (White et al., 1995; Tang et al., 1999). \(\alpha\)-defensins are characterized by \(C^1\cdotC^6\), \(C^2\cdotC^4\), \(C^3\cdotC^5\) connectivities; whereas \(\beta\)-defensins by \(C^1\cdotC^5\), \(C^2\cdotC^4\), \(C^3\cdotC^6\) connectivities while \(\theta\) defensins have a cyclic backbone and three disulfide linkages (White et al., 1995; Tang et al., 1999), as indicated in Chapter 1. No other pattern of disulfide connectivities have been observed for \(\alpha\)- and \(\beta\)-defensins. In this chapter the synthetic approach that was adopted to generate defensins and analogs with varying number of cysteines is described.

3.2 Choice of peptide sequences related to HNP-1, BNBD-12

In order to investigate the structural requirements for activity in \(\alpha\)- and \(\beta\)-defensins, the parent sequences whose structures had been determined by NMR and/or X-ray crystallography were chosen. The peptide sequences encompassed the triple stranded \(\beta\)-structure as well as the \(\beta\)-hairpin segments observed at the C-terminal ends of defensins as shown in Fig. 1.1. In case of peptide sequences corresponding to the native defensins, uniform as well as selective protection of cysteines was employed in order to examine the pattern of
disulfide connectivities formed, upon oxidation. Peptides corresponding to the \(\beta\)-hairpin region with truncations of the N-terminal segments were also synthesized in order to examine the functional role of the \(\beta\)-hairpin region.

Two and four cysteine residues, as they occur in the native sequences, were incorporated with a view to examine the requirement of disulfide bridges for stabilizing structure.

3.2.1. \(\alpha\)-defensin

\(\alpha\)-defensin HNP-1 is a member of a family of four structurally related peptides isolated from human neutrophils (Chapter 1, \(\alpha\)-defensins, page no.8; Selested et al., 1985; Lehrer et al., 1991; Lehrer et al., 1993).

The crystal structure for human neutrophil defensin HNP-3 was given by Hill et al., (1991). HNP-3 crystals were grown at room temperature by hanging drop method at a peptide concentration of \(\sim 20\) mg/ml in 0.01% acetic acid. The crystal structure revealed defensin to be an elongated, ellipsoidal molecule with over all dimensions of 26 Å by 15 Å by 15 Å as shown in Fig. 3.1A. HNP-3 appeared to be rigidly constrained by three disulfide bridges, dominated by a three-stranded antiparallel \(\beta\)-sheet structure involving 60% of all residues. The crystal structure also revealed that HNP-3 was in a dimeric state with each monomer linked to the other monomer directly through four hydrogen bonds and two indirectly through \(H_2O\) molecules. The hydrogen bondings are shown in Fig.3.1B. The dimeric association was further stabilized by hydrophobic interactions, especially between Cys\(^4\), Cys\(^{19}\), Tyr\(^{21}\) and Phe\(^{27}\) from each of the monomers. Later the solution structure of HNP-1 as determined by NMR (Pardi et al., 1992; Skalicky et al., 1994) suggested that the peptide adopts a structure similar to that of HNP-3. The NMR data suggested that HNP-1 also existed as dimer in solution.
Fig. 3.1. A. Stereoview of the polypeptide backbone defensin monomer, HNP-3, with four Arg side chains labeled and the three disulfide bridges shown in blue sticks. B. Pattern of hydrogen bonding in the defensin dimer. The disulfide bridges are shown as lightening bolts (Hill et al., 1991)
Thus, the crystal structure of human HNP-3 (Hill et al., 1991) and solution structures of rabbit defensin NP-5 and HNP-1 determined by NMR (Pardi et al., 1988; Pardi et al., 1992; Skalicky et al., 1994) together established that a triple stranded anti parallel \( \beta \)-sheet is the structural core in this family of peptides. It would not be unreasonable to assume that all \( \alpha \)-defensins have similar structures.

Based on NMR data, the structure proposed for HNP-1 consists of a stretch of double stranded anti parallel \( \beta \)-sheet in a hairpin conformation formed by the residues R14-C30. The hairpin contains a \( \beta \)-bulge formed by G17 residue, which is conserved amongst defensins from diverse vertebrate sources. Residues C2-I6 forms a small third strand. P7 exists as a cis peptide bond. The only difference observed between solution structure of HNP-1 and crystal structure of HNP-3, lies in the loop region involving residues 6-14 that is disordered in solution structure whereas X-ray studies indicate it to be ordered. Distribution of \( \beta \)-strands and turns in HNP-1 is shown in Fig. 3.2.

L HNP and L' HNP sequences correspond to native HNP-1 having the side chains of cysteines protected with Acm or Trt. In L HNP all the cysteine side chains are blocked by Acm, whereas in L'HNP dual cysteine protection group has been employed – Cys(Acm) [at C\(^1\) and C\(^6\) positions] and Cys(Trt) [at C\(^2\), C\(^3\), C\(^4\) and C\(^5\) positions].

Three residues from N-terminus and C\(^6\) are absent in peptide L26. In this sequence, four cysteines corresponding to C\(^2\), C\(^3\), C\(^4\), C\(^5\) are present.

The sequence L21 corresponds to the \( \beta \)-hairpin segment of HNP-1. The peptide has the two cysteines C\(^3\) and C\(^5\), C\(^4\) has been replaced with T.
Fig. 3.2. Primary structures of human neutrophil defensin HNP-1 and synthetic linear cysteine side-chain protected peptides corresponding to its different segments. The net positive charge at neutral pH are indicated in parantheses. The conformations of the different segments indicated in the HNP-1 sequence are as proposed by $^1$H NMR data. The charged residues are highlighted in bold.
3.2.2 β-defensins

Bovine neutrophil β-defensin, BNBD-12, is a member of a family of 13 structurally related peptides isolated from bovine neutrophils (Selested et al., 1993).

NMR studies (Zimmerman et al., 1995) indicated that BNBD-12 conserves the structural core of a triple stranded antiparallel β-sheet identical to that observed in α- defensins (Bach II et al., 1987; Pardi et al., 1988; Pardi et al., 1992), despite a different disulfide pattern. The N-terminal strand of BNBD-12, which is longer compared to HNP-1 was observed to be disordered. However, the authors have speculated the formation of a fourth strand by this region. As shown in Fig.3.3, the second strand (residues R22-C27) and the third strand (residues V32-R36) forms the β-hairpin secondary motif. The two strands being linked by a loop, residues F28-P31. Residues G10-I13 at the N-terminus forms a small third strand, followed by a Type II β-turn (residues I13-R16) and Type VI turn formed by residues P18-M21 with a cis proline P20. Both the turns are separated by cysteine at 17th position which forms the disulfide bridge with Cys35 yielding C3-C5 linkage. Distribution of β-turns and strands are shown in Fig.3.3.

The characteristic G-X-C motif observed to form a β-bulge and is conserved in defensins lies in the second strand. Most of the charged residues R22, R30, K33, R36 are positioned on the β-hairpin face except R7 and R16 which are present in turn 1 and 2 respectively. The positioning of these positively charged residues in the hairpin region forms an amphipathic structure that may be important for activity.
Fig 3.3. Primary structure of bovine neutrophil $\beta$-defensin, BNBD-12 and synthetic linear cysteine side-chain protected peptides corresponding to its different segments peptides. The net positive charges are indicated in parantheses. The conformations of the different segments indicated in the BNBD-12 sequence are as proposed by $^1$H NMR data.
L BNBD and L'BNBD are the linear forms of BNBD-12, with the cysteine side chains protected with Acm and/or Trt groups. In L BNBD all the cysteine thiols are protected with Acm, whereas in L'BNBD, Acm blocked cysteines are positioned selectively at C^1 and C^5 positions with Cys (Trt) at C^2, C^3, C^4 and C^6 positions.

Peptide L28 corresponds to the region of BNBD-12 with the triple stranded structure, the turns and the cysteines C^2, C^3, C^4 and C^6. All the cysteines being uniformly protected with Acm. In L'28 the cysteine side chains are protected with dual groups- Acm and Trt.

To the above mentioned structure in L28, the cysteines C^3 and C^6 has been removed giving rise to peptide L26.

Peptide L22 corresponds to a minimal sequence which has the β-hairpin structure at the C-terminal end of BNBD-12 and the cysteines C^3 and C^6.

3.3. Peptide synthesis and characterization:

All the peptides were synthesized on solid supports, based on Fmoc chemistry, using standard methods of activation, coupling and deprotection techniques (Atherton & Sheppard, 1989) as described in Experimental Procedures, section 2.2. Purity of crude linear peptides of HNP-1 series were assessed by FPLC using a reverse phase pep RPC HR (5/5) column and of BNBD-12 by HPLC using solvent system A: 0.1% TFA/H_2O and solvent B: 0.1% acetonitrile/H_2O. Peptides belonging to HNP-1 series LHNP, L26 and L21 eluted at 22.6% B, 30.5%B and 29.6%B. The HPLC profiles of the cysteine protected linear peptides are shown in Fig.3.4. The linear peptides of BNBD-12 series LBNBD, L28,
L26 and L22 eluted at 29.3%B, 28.2%B, 28%B, 24%B respectively as shown in Fig.3.5.

3.4. Formation of Disulfide Bridges:

There are two approaches for the formation of multiple intramolecular disulfide bridges in synthetic peptides — the single step approach where the peptide is oxidized after the simultaneous deprotection of all the cysteine protecting groups and the sequential approach where deprotection and oxidation are carried out in multiple steps (Andreu et al., 1994). In this study, both the approaches were followed.

3.4.1. Single Step Disulfide Bridge Formation

In this approach, all the disulfides are formed in a single step either by direct oxidation with an oxidant such as DMSO or air (Tam et al., 1991) or disulfide exchange with redox buffer systems.

Linear peptides LHNP, L26, L21 from HNP-1 series and LBNBD, L28, L26, L22 from BNBD-12 series were deprotected simultaneously using Hg(II) acetate treatment as described in Experimental Procedures, section 2.4, to yield free –SH peptides. Mercuric salts and 2-Mercaptoethanol were separated out from the peptides by gel filtration on a P-2 biogel column (55cm x 1cm), eluted with 0.01% TFA containing 50% acetonitrile/ H2O solvent system. Free thiol containing peptides so obtained were subjected to disulfide bridge formation in vitro by different methods summarized in Table 3.1. In all the cases, oxidation was monitored by Ellman's Reagent as described in Experimental Procedures, section 2.5.

i) Air oxidation:
Fig. 3.4. Reverse-phase HPLC of HNP-1 and analogs.
(a) L 21 (b) cy 21 (c) L 26 (d) L HNP-1. 10 μg of peptide samples were loaded and detected at 280 nm and 210 nm simultaneously at a flow rate of 0.5 ml/min. The dotted lines indicate the gradient used.
Fig. 3.5. Reverse-phase HPLC of cysteine protected linear analogs of BNBD-12.

(a) L 22  (b) L 26  (c) L 28  (d) L 38. 10 μg of peptide samples were loaded and detected at 280 nm and 210 nm simultaneously at a flow rate of 1 ml/min. The dotted lines indicate the gradient used.
Peptides were dissolved in aqueous solvent containing 1% methanol or acetonitrile at a concentration of 0.1-0.2mM. The pH was adjusted to 8.0 with NH₄OH and the solution was stirred for 18 hrs. Multiple disulfide containing peptides LHNP, L26, L28 and LBNBD upon oxidation formed aggregates, which remained insoluble till the addition of 2-mercaptoethanol or dithiothreitol (DTT). Even in the oxidation of single disulfide containing peptides L21 of HNP-1 series and L26, L22 of BNBD-12 series, aggregates were observed. Hence, the method was not used for oxidation of peptides.

ii) oxidation in aqueous DMSO (Tam et al., 1991):

DMSO was added to the reduced peptide solution at a final concentration of 20% by volume with constant stirring for 6 hrs. To avoid formation of aggregates, peptide concentration was kept as low as 0.2-0.4mM. The peptide solution was allowed to stir overnight, followed by gel filtration to get rid of excess DMSO. The buffer used was 50% acetonitrile/H₂O containing 0.005% TFA. Peptide stocks were lyophilized and used for further experiments.

Precipitation was negligible at the end of oxidation. For multiple cysteine containing peptides, the disulfide connectivities upon oxidation were established after enzymatic treatment of the oxidized products followed by mass spectral analysis and N-terminal sequencing, as summarized in Table 3.1. The disulfide bridges formed were the non-native connectivities. However, a single population was obtained and all the possible theoretical combinations were not formed. The connectivities in the four cysteine containing peptide, L26 (oxidation product is referred to as cy26') of HNP-1 series were observed to be C²-C³, C⁴-C⁵. In the six cysteine containing peptide LHNP (oxidation product is referred to as HNP-1') they were C¹-C³, C²-C⁴, C⁵-C⁶ as shown in Fig.3.6A & B. Similarly, from the
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Temperature</th>
<th>cosolvent</th>
<th>detergent</th>
<th>characteristic pattern observed in</th>
<th>aggregates</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>cy26'</td>
<td>cy28'</td>
</tr>
<tr>
<td>1. 20% DMSO/H2O</td>
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<td>nil</td>
<td>nil</td>
<td>C2 C3 C4 C5/C6*</td>
<td>no</td>
</tr>
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<td>nil</td>
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<td>no</td>
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<td>5% TFE</td>
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<td>- do -</td>
<td>less</td>
</tr>
<tr>
<td>4. 20% DMSO/H2O</td>
<td>4°C</td>
<td>5% TFE</td>
<td>nil</td>
<td>- do -</td>
<td>more</td>
</tr>
<tr>
<td>5. 20% DMSO/H2O</td>
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<td>nil</td>
<td>6M GuHCl</td>
<td>- do -</td>
<td>no</td>
</tr>
<tr>
<td>6. A</td>
<td>4°C</td>
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<td>nil</td>
<td>not checked</td>
<td>not checked</td>
</tr>
<tr>
<td>7. B</td>
<td>4°C</td>
<td>nil</td>
<td>nil</td>
<td>mixed disulphide with GSH</td>
<td>less</td>
</tr>
<tr>
<td>8. Air oxidation</td>
<td>RT</td>
<td>NH4CO3</td>
<td>nil</td>
<td>not checked</td>
<td>high</td>
</tr>
</tbody>
</table>

Buffer A contains 5mM cysteine, 5mM EDTA and 0.01M NaPi
Buffer B contains GSH: GSSG at 10mM:1mM, 1mM EDTA, pH 7.0

* C2-C3, C4-C5 represent for cy26' and C2-C3, C4-C6 represent for cy28'.

Table 3.1. Conditions used for disulfide bridge formation by single step oxidation
**Fig. 3.6A.** Schematic representation of enzymatic treatment and assignment of disulfide linkages in cy26'
Fig. 3.6B. Schematic representation of enzymatic treatment and assignment of disulfide linkages in HNP-1'
linear peptides corresponding to segments of BNBD-12, the connectivities were $C_2$-$C_3$, $C_4$-$C_6$ where 4 cysteines were present and $C_1$-$C_3$, $C_2$-$C_4$, $C_5$-$C_6$ where 6 cysteines were present. The data are summarized in Fig.3.7A & B. Thus L28 and LBNBD upon oxidation by this method is referred to as cy28' and BNBD-12' respectively.

iii) DMSO with organic solvent:

Since Goodman's group first used trifluoroethanol (TFE) solvent to study peptide conformation in 1962 (Goodman and Listowsky, 1962), TFE has been widely used as a structure inducing cosolvent for small protein fragments and flexible peptides (Kippan and Fersht, 1995; Hamada et al., 2000). In this method, DMSO was added to the final concentration of 20% by volume as described above, containing 5% TFE. The mixture was stirred overnight. This method resulted in formation of insoluble aggregates. In the soluble fraction, the small amount of peptides did not have disulfide connectivities as in the native defensins, as judged by enzymatic digestions. The connectivities were similar to that obtained in aqueous DMSO. The connectivities observed in four and six cysteine containing peptides corresponding to HNP-1 and BNBD-12 series are summarized in Table 3.1.

iv) Using the denaturant 6M Gu HCl (Tam et al., 1991):

The reduced peptides obtained after deprotection of Acm protected cysteines, were dissolved in 6M GuHCl containing 20% DMSO and stirred for 10-12 hrs. Subsequently, peptides were gel filtered over a P-2 biogel column to get rid of denaturants. By this approach, though aggregate formation was reduced, the peptides did not have the disulfide connectivities observed in native defensin. In case of BNBD-12, the obtained disulfide connectivities are shown in Fig.3.7A & B.
Fig. 3.7A. Schematic representation of enzymatic treatment and assignment of disulfide bridges in cy28.
Theoretical  
(M+H)<sup>+</sup>  
(M+H)<sup>+</sup>  
Disulfide linkage

<table>
<thead>
<tr>
<th>Fragments obtained</th>
<th>Theoretical 1594</th>
<th>Observed 1595</th>
<th>Disulfide linkage</th>
<th>Fragments obtained</th>
<th>Theoretical 1367</th>
<th>Observed 1368</th>
<th>Disulfide linkage</th>
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<td>1368</td>
<td>C&lt;sub&gt;3&lt;/sub&gt; C&lt;sub&gt;4&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NGGVC&lt;sup&gt;2&lt;/sup&gt;PIR</td>
<td>1807.4</td>
<td>1808</td>
<td>C&lt;sub&gt;1&lt;/sub&gt; C&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;3&lt;/sub&gt; C&lt;sub&gt;4&lt;/sub&gt; C&lt;sub&gt;5&lt;/sub&gt; C&lt;sub&gt;6&lt;/sub&gt;</td>
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<td></td>
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</tr>
<tr>
<td>QIGTC&lt;sup&gt;4&lt;/sup&gt;F</td>
<td>1746</td>
<td>1746</td>
<td>C&lt;sub&gt;3&lt;/sub&gt; C&lt;sub&gt;4&lt;/sub&gt; C&lt;sub&gt;5&lt;/sub&gt; C&lt;sub&gt;6&lt;/sub&gt; or</td>
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<td>6M GuHCl</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;3&lt;/sub&gt;PVPMR</td>
<td>1388.7</td>
<td>1389</td>
<td>C&lt;sub&gt;2&lt;/sub&gt; C&lt;sub&gt;4&lt;/sub&gt;</td>
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<td></td>
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</tr>
</tbody>
</table>

Fig. 3.7 B. Schematic representation of enzymatic treatment and assignment of disulfide bridges in BNBD-12'
However, in HNP-1 the connectivities were identical to that formed with 20% DMSO, Table 3.1.

v) Using Redox Buffers (Rothwarf and Scheraga, 1993; Annis et al., 1997; Kuhelj et al., 1999):

Multiple thiol containing proteins have been oxidized by redox buffers of suitable composition. Redox buffers changes the mechanism from direct oxidation to thio-disulfide exchange (thiolate-intermediate), which facilitates the reshuffling of misfolded analogs to the correct disulfide bridged natural ones.

The main chemical reaction involved in thiol/disulfide exchange (Hantgan et al., 1974; Gilbert, 1990; Wedemeyer et al., 2000) is represented as:

\[ R_1S^- + R_2SSR_3 \rightarrow R_2S^- + R_1SSR_3 \]

in which the thiolate anion \( R_1S^- \) displaces one sulfur of the disulfide bond \( R_2SSR_3 \). Disulfide bonds are formed and reduced by two such thiol/disulfide exchange reactions with a redox reagent, the first of which involves the formation of a *mixed disulfide bond* between the protein and the redox reagent.

In this study, two composition have been employed to refold the multiple disulfide containing peptides. First redox mixture contained cysteine 5mM, EDTA 5mM and 0.01M sodium phosphate (NaPi) buffer (Annis et al., 1997; Kuhelj et al., 1999). However it resulted in formation of aggregates, which could not be solubilised until further addition of DTT. The other redox buffer had reduced glutathione (GSH) to oxidized glutathione (GSSG) at 10mM:1mM ratio and EDTA 1mM, pH adjusted to 7.0 (Hantgan et al., 1974; Annis et al., 1997). Peptides were allowed to fold at room temperature and 4°C for 16 hrs. Compared to the former the latter composition resulted in less aggregate formation. However,
MALDI-tof mass spectroscopy revealed that the peptides formed mixed disulfide with GSH. Thus, for peptides HNP-1', cy26', BNBD-12' and cy28' calculated [M+H]^+ and observed [M+H]^+ were 3443 and 3750; 3004 and 3011; 4043 and 4350; 3157 and 3465 respectively, where the [M+H]^+ of adduct GSH is 307.33.

Amongst all the conditions studied for disulfide bridge formation by single step process, aggregate formation was practically absent in 20% DMSO in H_2O. Multiple disulfide containing analogs namely, cy26' and three disulfide containing HNP-1 could not be characterized by either of HPLC/FPLC presumably due to aggregation. A variety of solvent systems like acetonitrile, methanol, isopropanol were employed for HPLC/FPLC analysis, to characterize the oxidized products. However the single disulfide containing analog cy21 separated well in FPLC at a 41.8%B as shown in Fig.3.4. The oxidized products of BNBD-12 series namely, cy22, cy26, cy28 and BNBD-12 containing single, two and three disulfide bridges were characterized by HPLC as shown in Fig.3.8. The peptides cy22, cy26, cy28' and BNBD-12' eluted at 23.4%B, 29.5%B, 29.2%B and 29.6% B respectively. MALDI-TOF mass spectrometry and N-terminal sequencing of the peptides confirmed the sequence, without any chemical modifications.

3.4.2. Two Step Selective Disulfide Bridge Formation

In this approach, dual cysteine protecting groups have been employed. Thus peptides with six cysteines were selectively protected with two different blocking groups – four with trityl and two with acetamidomethyl(Hiskey, 1981; Barany and Merrifield, 1980). Disulfide bridge formation was performed in a two step procedure as shown schematically in Fig.3.9 and Fig.3.10. In the first step, cleavage mixture removed Trt along with other side groups being acid labile, except the Acm. The four cysteines with free –SH groups were then allowed to fold in 20%
Fig. 3.8. Reverse-phase HPLC of (a) cy 22 (b) cy 26 (c) cy28' after oxidation in 6M Gu HCl containing 20% DMSO (d) BNBD-12' after oxidation in 6M Gu HCl containing 20% DMSO. Native cy28 and BNBD-12 also eluted at the same %B solvent. The gradient used is indicated by the broken line (% acetonitrile). 10μg of the peptide samples were injected and detected at 280nm and 210 nm simultaneously at a flow rate of 1ml/min.
Fig. 3.9. Schematic representation of oxidation steps to form disulfide bridges in HNP-1 and variants. A represents 'single step oxidation' and B represents 'selective oxidation'. L 21 was oxidised in single step.
Fig. 3.10. Schematic representation of disulphide bridge formation in BNBD-12 and variants. (A) represents 'single step oxidation' and (B) represents 'selective oxidation' for three and two disulfide containing peptides. L 26 and L 22 containing single disulfide bridges were oxidised in a single step.
DMSO / H₂O with constant stirring for 4-5 hrs. Peptide concentration being 0.1-0.2mM. Following this, peptides were gel filtered to get rid of DMSO.

In the second step, the Acm protecting groups were concomitantly deprotected and oxidized by I₂ (Annis et al., 1997) to yield the third disulfide bridge.

Peptides L'HNP (having dual cysteine protecting groups) was oxidized following this strategy which resulted in the formation of disulfide connectivities between C₁-C₆, C₂-C₄, C₃-C₅ as indicated by enzymatic digestions in Fig.3.11. Peptides L'BNBD and L'28 were also oxidized by this strategy resulting in formation of the desired product with the connectivities C₁-C₅, C₂-C₄, C₃-C₆ referred to as BNBD-12 and C₂-C₄,C₃-C₆ as cy28 as indicated by enzymatic digestions, Fig.3.12A & B.

3.5. Discussion:

Defensins, composed of six cysteines are ideal molecules to investigate how disulfide connectivities are established in vitro, correlating the type of disulfide linkages with structure and effects on activity.

In this study, disulfide formation in two different class of defensins namely, α- and β-defensin have been studied under different oxidation conditions. The strategy directed to cyclise the peptides in a single step resulted in the formation of either insoluble aggregates or peptides with disulfide bridges different from those observed in the native defensins. In rabbit defensin NP-1, disulfide bridge formation as observed in the native peptide has been achieved by air oxidation (Gururaj Rao et al., 1992). More recently in rabbit defensin RK-1, disulfide bridges as in native form has been achieved by 20% aqueous DMSO containing 5% aqueous acetic acid (Dawson et al., 2000). Tam et al.,(1991) in their
AC'TYC2RIPAC3IAGERRYGTC'4TYQGRWLWAC5C6

selective oxidation

C1(Acm) C2 C3 C4 C5 C6(Acm) or

C1(Acm) C2 C3 C4 C5 C6(Acm) or

C1(Acm) C2 C3 C4 C5 C6(Acm)

Trypsin digestion

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<thead>
<tr>
<th>Fragments obtained</th>
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<th>Obtained ([M+H]^+)</th>
<th>Disulfide linkage</th>
</tr>
</thead>
<tbody>
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<td>3214.3</td>
<td>3237*</td>
<td>C1(Acm) C2 C3 C4 C5 C6(Acm)</td>
</tr>
<tr>
<td>AC1(Acm)YC2RIPAC3IAGEREYGTCT4TYQGR AFC5C(Acm)</td>
<td>3167</td>
<td>3190*</td>
<td></td>
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</tbody>
</table>

C2R
gtc4tyqgr

1172
1172

Ac3IAGER

LWAFc5C(Acm)

1740
1741

Ac3IAGER

AFc5C(Acm)

1441
1442

Fig. 3.11. Schematic representation of enzymatic treatment and assignment of disulfide linkages in HNP-1
Selective oxidation

C\(^2\)(Trt) C\(^3\)(Acm) C\(^4\) (Trt) C\(^6\)(Acm)

Trypsin digestion

<table>
<thead>
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<th>Fragments</th>
<th>Theoretical (M+H(^+))^(^+)</th>
<th>Obtained (M+H(^+))^(^+)</th>
<th>Disulfide linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>GVC(^2)PIR</td>
<td>1636.2</td>
<td>1637</td>
<td></td>
</tr>
<tr>
<td>QIGTC(^4)FGR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(^3)PVPRM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(^6)R</td>
<td>977.3</td>
<td>980</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3.12A. Schematic representation of enzymatic treatment and assignment of disulfide bridges in cy28.
Selective oxidation

\[
\begin{align*}
C^1& <t C^2 \quad \text{or} \quad C^3 \quad C^4 <t C^5 (\text{Acm}) \quad C^6 \\
C^1 & <t C^2 \quad \text{or} \quad C^3 \quad C^4 <t C^5 (\text{Acm}) \quad C^6 \\
C^1 & <t C^2 \quad \text{or} \quad C^3 \quad C^4 <t C^5 (\text{Acm}) \quad C^6
\end{align*}
\]

Trypsin digestion

<table>
<thead>
<tr>
<th>Fragments obtained</th>
<th>Theoretical (M+H(^+)) (^t)</th>
<th>Observed (M+H(^+)) (^t)</th>
<th>Disulfide linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGGVC^2IPIR</td>
<td>2131.6</td>
<td>2132</td>
<td></td>
</tr>
<tr>
<td>QIGTC^4FGRPVK</td>
<td>1476</td>
<td>1477</td>
<td></td>
</tr>
<tr>
<td>C^3PVPMR</td>
<td>1476</td>
<td>1477</td>
<td></td>
</tr>
<tr>
<td>PVKC^5(\text{Acm})C^6R</td>
<td>1476</td>
<td>1477</td>
<td></td>
</tr>
</tbody>
</table>

Fig.3.12B. Schematic representation of enzymatic treatment and assignment of disulfide bridges in BNBD-12
earlier studies have reported the formation of disulfide bridges in synthetic HNP-1 as in the native peptide by using aqueous DMSO although the yield appeared to be low. However, Raj et al., (2000) have reported native disulfide bridge formation in HNP-1 by using three different pairs of protecting groups for the cysteine thiols. Results from this work shows that air oxidation has resulted in formation of mostly insoluble aggregates. Although aqueous DMSO has been efficient in minimizing aggregates and intermediates with redox reagents were absent, it resulted in formation of non-native disulfide bridges. The two disulfide containing peptide cy26' corresponding to HNP-1 series, upon oxidation formed "near-neighbour" disulfide bridges C2-C3, C4-C5 than the "native linkage" C2-C4, C3-C5. Similarly, in cy28' corresponding to BNBD-12 series, the linkages observed were C2-C3, C4-C5 instead of the native disulfide bridging pattern C2-C4, C3-C6. The three disulfide containing peptides HNP-1' and BNBD-12' were C1-C3, C2-C4, C5-C6 linked instead of C1-C6, C2-C4, C3-C5 and C1-C5, C2-C4, C3-C6 connectivities respectively. Only in the case of the peptide corresponding to linear BNBD-12, many of the theoretically possible disulfide connectivities were observed when oxidation was carried out in the presence of GuHCl.

The study indicates that in three disulfide containing peptides, the adjacent cysteines at the C-terminus i.e., C5 and C6 always formed disulfide bridges irrespective of the oxidation conditions. Similarly, at the N-terminus the linkages were formed between C1 and C3. This observed pattern of disulfide linkage was observed under all the conditions studied, though formation of aggregates and intermolecular disulfide bridge formation was minimized using denaturants. Even the presence of structure promoting organic solvents during the refolding conditions did not yield peptides with the native disulfide connectivities. However, under the conditions where aggregate formation has been minimized by
using denaturants like GuHCl or DMSO, the peptides were mostly a ‘single-
population’ of non-native disulfide bridged patterns and not a combination of all the
theoretically possible linkages.

In order to form disulfide bridges as in native defensins, two step regioselective synthesis was adopted (Spetzler et al., 1994; Yang et al., 1994; Annis et al., 1997; Tam and Lu, 1998). In this method while one pair of cysteines remain blocked, the two pairs of cysteines are allowed to bridge. The four cysteines can pair up in three possible ways leading to a scrambled population. However, in spite of other possibilities, the correct pairs were formed. The remaining third pair of cysteine are deprotected and oxidized by using I₂ oxidation.

Synthesis of linear sequences of HNP-1 and following oxidation subsequent fragmentation with endopeptidases followed by their characterization, together shows that the synthesized analog has exactly the same connectivities as of the native HNP-1 i.e., C₁-C₆, C²-C⁴,C³-C⁵. Similarly, in case of BNBD-12 and its analog cy28, the disulfide bridges formed were similar to as observed in the native BNBD-12 i.e C₁-C₅,C²-C⁴,C³-C₆. Further structure-function studies were carried out by using all the linear peptides, peptides with disulfide connectivities as in native defensins and those with different of HNP-1 and BNBD-12 series.