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SPECTROSCOPIC INVESTIGATION OF SELF-ASSEMBLING PROPERTIES OF TYROCIDINES B AND C: A NOVEL GROUP OF PEPTIDES AGAINST FOODBORNE PATHOGENS

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The self-assembling properties of two of the major tryptophan containing peptides of the Tyrocidine group, TrcB and TrcC were investigated in aqueous and membrane-mimicking environment. The critical self-assembling concentrations of the two peptides (csac) were determined using an extrinsic fluorophore 1, 8-anilinonaphthalenesulfonamide (ANS). Steady-state fluorescence and circular dichroism studies in trifluoroethanol and sodium dodecyl sulphate indicate the disruption and formation of new intra/inter molecular hydrogen bonds in the tyrocidines. Calcium ions contribute in the H-bond alterations in the secondary structures of the peptides.

Keywords: Tyrocidine, Self-assembly, Critical self-assembling concentration, Fluorescence, Circular dichroism

INTRODUCTION
Antimicrobial peptides have been considered as a potential source of a new class of antibiotics because of their broad spectrum of activity against bacteria, some fungi and parasites (Hwang and Vogel, 1998; van’t Hof et al., 2001; Hancock and Patrzykat, 2002; Lee, 2002; Vizioli and Salzet, 2002; Jønsen et al., 2006; and Rautenbach et al., 2007). A large degree of structural diversity is found in the collection of antimicrobial peptides, but they share one important feature; their amphipathic character. Consequently, many of these antimicrobial peptides are commonly referred to peptide antibiotics. They cause disruption of function of cell membrane and other intracellular membranes which often leads to rapid death of the organism. The basis for selectivity appears to be related to the composition of the target membrane. The rapid killing kinetics, multiple targets and lack of natural resistance towards antimicrobial peptides most probably led to the evolutionary selection of this abundant group of defense molecules. Accordingly, the molecular basis of their action is of considerable interest and requires to be elucidated.

Dubos and Hotchkiss (Dubos, 1939a and 1939b) found potent antimicrobial activity in the fermentation broth of Bacillus brevis, now classified as Bacillus aneurinolyticus. This activity was due to a diverse group of antimicrobial peptides collectively known as the tyrothricin complex (Hotchkiss, 1941; Hotchkiss and Dubos, 1941; and Tang et al., 1992) which is composed of the basic, cyclic tyrocidines and neutral, linear gramicidins. Tyrothricin was one of the first antibiotics in clinical use, albeit as topical antibiotic (Van Epps, 2006). The primary structures of 28 tyrocidines and analogues have been determined and a studies by Gibbons et al. (1975) and Kuo and Gibbons (1980) suggest that these cyclic decapetides assume antiparallel β-pleated sheet structures. The tyrocidines contain one of the pentapeptide repeats of gramicidin S, Val-Orn-Leu-Pro-...
In some tyrocidines the Orn residue is substituted with Lys. The other five residues in the tyrocidines differ only by conservative substitutions in the aromatic dipeptide unit. The tyrocidines are known to possess broad spectrum antimicrobial activity toward Gram-positive and Gram-negative bacteria (Dubos and Dubos, 1939; and Dubos and Hotchkiss, 1941) and parasites, notably the malarial parasite *Plasmodium falciparum*.

Since the antibacterial studies done early in last century on the tyrocidine mixture, only a few follow up molecular and activity studies were conducted. These studies mostly involved investigation of the structure (Laiken et al., 1969; Gibbons et al., 1975; Kuo and Gibbons, 1980; and Tang et al., 1992) and aggregation properties (Laiken et al., 1969 and 1971) of the tyrocidines, generation of synthetic analogue libraries (Qin et al., 2003; and Marques et al., 2007) and the antiplasmodial activity of the major tyrocidines. We decided to focus on two major tyrocidines, TrcB and TrcC (Illustration 1) with one and two tryptophan residues respectively, their structural variations on aggregation/self-assembly. Both the antilisterial and antiplasmodial activity was found to be highly dependent on peptide identity and self-assembly (Spaethelf and Rautenbach, 2009). The antilisterial activity of the tyrocidines was shown to be associated with increased self-assembly within a membrane-like environment whereas the antiplasmodial activity of the tyrocidines was shown to be associated with reduced self-assembly within a membrane-like environment. In order to know the behavior of the peptides in different environments, a detailed study of self-assembling properties of the two tyrocidines, TrcB, and TrcC is performed in aqueous and membrane-mimetic environment like trifluoroethanol and in aqueous solution of Sodium Dodecyl Sulphate (SDS).

The broad spectrum antibacterial activity, speed of action and reduced likelihood of resistance development toward antimicrobial peptides suggest that these compounds may be useful as bio-preservatives (Hancock and Lehrer, 1998). Nisin, pediocin, and bacteriocins produced by Lactic Acid Bacteria (LAB), have been shown to effectively inhibit *L. monocytogenes* growth in various food products and are currently accepted for use as food preservatives (Benkerroum and Sandine, 1988; Montville and Chen, 1998; Brul and Coote, 1999; Nes and Holo, 2000; Cleveland et al., 2001; and Gravesen et al., 2004). In addition to the challenge of resistance-development, control of *L. monocytogenes* in food is complicated by its high temperature, salt and pH tolerance (Pearson and Marth, 1990; Parente et al., 1998; and Gandhi and Chikindas, 2007). The ability of *L. monocytogenes* to grow under high salt conditions pose a significant problem for the application of antimicrobial peptides as bio-preservatives since the activity of most antimicrobial peptides is reduced by the presence of cations such as Na\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) (Lehrer et al., 1988; Skerlavaj et al., 1990; Cociancich et al., 1993; Yamauchi et al., 1993; Broekaert et al., 1995; Bals et al., 1998; and Bowdish et al., 2005). Divalent cations, especially Ca\(^{2+}\), lead to a dramatic reduction in antibacterial activity of various antimicrobial peptides, including nisin and pediocin. The inhibition of antimicrobial peptide activity by cations may be due to either increased aggregation induced by cations (Urrutia et al., 1989; and Montville and Chen, 1998) which would decrease the number of active peptide, or inhibition of membrane-binding, probably by shielding electrostatic interaction between the peptide and the target membrane (Cociancich et al., 1993; Montville and Chen, 1998; and Rydlo et al., 2006). Similarly, some preliminary data indicated that the bioactivity of the tyrocidines to be highly sensitive to environmental conditions, notably the presence of calcium. The antilisterial activity, as well as the mode of action, of the tyrocidines was also found to be highly sensitive to tyrocidine-Ca\(^{2+}\) complexation. It may be hypothesised that tyrocidine activity and mode of action is

**Illustration 1: Primary Structure of Tyrocidines; the Amino Acid Abbreviations in Brackets Indicate the Various Tyrocidine Analogues, TrcB: Cyclo-(VOLfPWfNQY), TrcC: Cyclo-(VOLfPWwNQY)**
modulated by a critical play-off between self-assembly, cation-complexation and membrane-interaction. As Ca\(^{2+}\) ions are found to play an essential role in biological activity of the peptides, different concentrations of Ca\(^{2+}\) ions are used in the current study to elucidate the effect of the ion on the structural assembly of the peptides in aqueous and membrane-mimetic environment. Investigation of the influence of cations on the antilisterial activity of the tyrocidines may thus provide valuable information for development of the tyrocidines for use as bio-preservatives for food.

**EXPERIMENTAL**

The tyrothricin (from *Bacillus aneurinolyticus*), 1, 8-anilinonaphthalene sulfonamide (ANS) was supplied by Sigma (USA). Sodium dodecyl sulphate was obtained from Melford (UK). Calcium Chloride was obtained from BDH Chemicals (England). Diethyl ether and acetone were supplied by Saarchem (Krugersdorp, South Africa). Acetonitrile (ACN) (HPLC-grade, far UV cut-off) was supplied by Romill Ltd. (Cambridge, UK). Trifluoroethanol (TFE) was obtained from Fluka. Waters-Millipore (Milford, USA) supplied the Nova-Pak C18 (5 µm particle size, 60 Å pore size, 7.8 mm x 300 mm) semi-preparative HPLC column. Analytical grade water was prepared by filtering water from a reverse osmosis plant through a Millipore Milli-Q water purification system (Milford, USA).

The two major tyrocidines were isolated from commercially obtained tyrothricin. The crude fractionation was performed by organic extraction, as described by Hotchkiss and Dubos (1941), with some modifications. Briefly, the tyrothricin in dry powder form was washed three times with equal volumes ether/acetone (1:1, v/v) and the insoluble fraction/precipitate containing the tyrocidines was collected by centrifugation and dried under vacuum. The tyrocidines in the tyrocidine fraction were subsequently purified by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), according to the methods described by Rautenbach et al. (2007). The collected fractions were subjected to analytical RP-HPLC, time-of-flight electrospray mass spectrometry (TOF-ESMS), and tandem mass spectroscopy (MS-MS) in order to determine the degree of purity of each fraction and to identify the purified product.

The absorption and steady-state fluorescence spectra were recorded on a UV-Visible spectrophotometer (Cary100, Varian) and a spectrofluorimeter (Perkin Elmer), respectively. The fluorescence spectra were corrected for the instrumental response. The peptide concentrations used for absorption and emission ranged from 0-100 mM. The sample solutions were excited at 280 nm. Circular Dichroism (CD) spectra were recorded in Chirascan Plus (Applied Photophysics). In case of recording of CD spectra, the concentrations of the tyrocidines were increased to 200 mM.

**RESULTS AND DISCUSSION**

**Determination of Critical Self-Assembling Concentration**

1, 8-anilinonaphthalene sulfonamide (ANS) is a successful probe for measuring the hydrophobicity of its surrounding environment. Addition of TrcB and TrcC to the aqueous solution of ANS (80 µM) exhibited a red shift in the absorption spectra and a blue-shift in the emission spectra of ANS (Figure 1). The red shift in the absorption spectra signifies that ANS moves into the non polar region where the ground state becomes less stabilized than the previous existing ground state. The enhancement and blue-shift in the emission spectra further supports the fact that the probe (ANS) moves into a hydrophobic region which is possibly created due to the self-assembling of the tyrocidines. Critical self-assembling concentrations (csac) for both the peptides are determined from the inflexion point of the fluorescence intensity versus concentrations plots of the peptides (Figure 2). The values obtained for TrcB and TrcC are 33 µM and 50 µM.
μM respectively. The order in csac is observed as: Trc C>Trc B, which follows the activity order towards Gram-positive bacteria, *M. luteus* and strains of *L. monocytogenes* B73 and B73-MR1.

**Effect of Membrane-Mimicking Environments (Trifluoroethanol and Sodium Dodecyl Sulphate)**

**Trifluoroethanol**

**Absorption:** A gradual decrease in the optical density was observed for the peptides on addition of trifluoroethanol (TFE) to their aqueous solutions. It was further noticed that the nature of the absorbance curve of these peptides change at higher concentrations of TFE (Figure 3) which suggested that at higher concentrations of TFE, structural changes occur in the peptides.

**Emission:** The emission spectra of Trc B and Trc C (~ 10 μM) are recorded in acetonitrile, trifluoroethanol and water. A red-shift of the emission maxima was observed for the tryptophan fluorophore with increase in polarity of the media (Figure 4). The red shift of the tryptophan fluorophore for the tyrocidines in the three different solvents as mentioned above is due to the increasing polarity of the media.

In the emission profile of the individual peptides, it was observed that upon gradual addition of TFE to the aqueous solution of the peptides (10 μM), the emission band of tryptophan at 350 nm undergoes quenching whereas an emission band is visible at 308 nm (Figure 5). The significant quenching of the tryptophan emission at 350 nm suggests
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Fluorescence for these peptides can be attributed to the H-bonding interactions. The formation of higher-ordered structures is also supported from Circular Dichroism (CD) spectra of these peptides in TFE. The emission band at 308 nm is attributed to tyrosine residue (Ugurbil and Bersohn, 1977).

**Circular Dichroism:**
Both the peptides TrcB and TrcC show similar CD spectra in acetonitrile-water solvent system (Figure 6) at higher concentrations (195 µM) which suggests that their self-assembling behavior is similar in that concentration range. The spectrum at higher concentrations of peptides is characterised by two band minima at 205 nm and 216 nm while TrcC also shows a minima around 230 nm for the aromatic tryptophan residue. The ratio of the two minima is not close to 1 unlike α-helical structures rather the spectral behavior corresponds to β-sheeted structure with β turns. But at lower concentrations, TrcC and TrcB have different spectra which throw light on their affinity to aggregate. At a much lower concentration (95 µM) TrcC shows a single band minimum at 222 nm which corresponds more to a random orientation of the monomeric peptide, but TrcB has two minima even at that low concentration (Figure 6) indicating an aggregated state for TrcB. The concentration behavior of the two peptides suggests that TrcB has higher affinity to aggregate as compared to TrcC and the statement is also supported from the earlier fluorescence studies as TrcB has a lower c_sac value than TrcC.

**Figure 4:** Fluorescence Spectra of TrcC in Different Environments

**Figure 5:** Emission Spectra of TrcC (10 µM) in Presence of TFE at $\lambda_{ex} = 280$ nm, Concentrations of TFE are Indicated in Legends

**Figure 6:** CD Spectra of TrcB and TrcC at their Different Concentrations in Acetonitrile-Water Medium

that there are new interactions which increase the nonradiative deactivation pathway in the system. These class of antimicrobial peptides self-assemble in presence of TFE as the latter is known to provide membrane-mimetic environment inducing secondary structures (Nelson and Kallenbach, 1989; and Lehrman et al., 1990). The induction of higher-ordered structures with addition of TFE may be via formation of new intramolecular/intermolecular H-bonds. The indole group of the tryptophan moiety in peptides can participate in hydrogen exchange as observed in other cases (Hu and Cross, 1995). The quenching of tryptophan fluorescence for these peptides can be attributed to the H-bonding interactions. The formation of higher-ordered structures is also supported from Circular Dichroism (CD) spectra of these peptides in TFE. The emission band at 308 nm is attributed to tyrosine residue (Ugurbil and Bersohn, 1977).

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On addition of TFE to TrcC the band at 205 nm starts to develop while 222 nm band blue-shifts to 216 nm (Figure 7a). On the other hand, in TrcB addition of TFE enhances the 205 nm band (Figure 7b). The formation of the new band at 205 nm for TrcC and enhancement of the same band (i.e., 205 nm band) for TrcB with addition of TFE suggests that the 205 nm band is associated with self-assembly/aggregation process. TFE is known to induce secondary and higher structural forms in peptides and proteins via H-bond formation. The observations further substantiate that (1) the 205 nm band is related with intramolecular H-bond interactions and (2) the band around 216 nm is associated with backbone structure as it almost remains intact. The increase or decrease in the ellipticity of the spectra is associated with H-bond formation and disruption respectively.

**Sodium Dodecyl Sulphate:** The addition of SDS to the aqueous solution of the peptides hardly changes the absorption spectra of the peptides (Figure 8a) but the emission spectra (Figure 8b) show significant changes similar to the observations recorded with addition of TFE. The quenching of the 350 nm band for tryptophan fluorescence hint to the fact that like TFE, SDS also induces...
certain H-bond interactions due to which the radiative processes are quenched. The tyrosine emission at 308 nm is consequently observed on quenching of the tryptophan fluorescence (Figure 8b).

The 205 nm band in CD spectra (Figure 9) disappears for both the peptides TrcB and TrcC in presence of SDS at a concentration of ~ 9 mM which is a concentration higher than the CMC of SDS. In micellar environment, the spectra of TrcB and TrcC look quite similar with minima around 220 nm and a dip at 230 nm.

The disappearance of the 205 nm band in the CD spectra (Figure 9) for both the peptides TrcB and TrcC in presence of SDS at a considerably higher concentration implies that SDS micelles cause structural disorganization, break the higher order structures via disruption of H-bonds and the peptides tend to form monomeric structures. This observation is consistent with the earlier observations and interpretation where the 205 nm band is being correlated with the self-assembly formation. Thus in presence of SDS, the spectra of both the peptides look almost identical with minima around 220 nm (characteristic of the peptide backbone structure) and a dip for the aromatic tryptophan residue at 230 nm, thus representing peptides under random orientation.

**Circular Dichroism:** In aqueous media the ellipticity of the CD spectra for TrcB sharply decreases with addition of Ca$^{2+}$ ions (Figure 10). While in TFE, the change in the spectra is negligible in presence of Ca$^{2+}$ ions. The effect of Ca$^{2+}$ ions on TrcC aggregates is similar to TrcB (Figure 11) in a particular environment.

The sharp decrease in the ellipticity of the CD spectra for TrcB in aqueous media with addition of Ca$^{2+}$ ions reflects that the intramolecular H-bonds gets disrupted by the observations suggest that the divalent metal ion like Ca$^{2+}$ ions hinder the self-assembling process of the peptides and the peptides restrict themselves in lower order aggregates. Hence there is almost no quenching of tryptophan emission at 350 nm.

**Effect of Calcium Ions (Calcium Chloride)**

**Emission:** The addition of Ca$^{2+}$ ions negligibly quenched the tryptophan emission of the peptides in the environment of trifluoroethanol/deuterated trifluoroethanol. The
incorporation of the metal ion. But in TFE, the Ca\textsuperscript{2+} ions could not bring much change which suggests that the H-bonds and thereby the aggregation is much stronger in TFE. The changes in TrcC is similar to that of TrcB with Ca\textsuperscript{2+} ions which indicate that in TrcC also Ca\textsuperscript{2+} ions can break the self-assembling when in aqueous media but not in TFE. Thus the intramolecular H-bonds of the peptides are strongly susceptible to Ca\textsuperscript{2+} ions in aqueous media while less susceptible to changes in membrane-mimetic trifluoroethanol environment.

The change in ellipticities at the two different wavelengths (205 and 216 nm) for both tyrocidines are plotted against concentration of the Ca\textsuperscript{2+} ions (Figure 12) the slope gives the measure of intramolecular H-bond disruption.

Table 1: Slope of the Curves for TrcB and TrcC in Two Different Environments

<table>
<thead>
<tr>
<th></th>
<th>acn - water</th>
<th>acn - tfe</th>
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<tbody>
<tr>
<td>TrcC</td>
<td>0.032</td>
<td>0.045</td>
</tr>
<tr>
<td>TrcB</td>
<td>0.073</td>
<td>0.015</td>
</tr>
</tbody>
</table>

The slope of the curves from Table 1 further supports that the intramolecular H-bonds being stronger in TFE, the Ca\textsuperscript{2+} ions act as weak chaotropic agent here as the slope gives an account of the stability of the intramolecular H-bonds for the two peptides. The different effect of Ca\textsuperscript{2+} ions
and thus the different slopes in the two media for the peptides also possibly indicate that the self-assembling behaviour of the peptides differ from one media to another.

CONCLUSION

The self-assembling behaviour of the tyrocidines viz. TrcB and TrcC differ in different environments. The critical self-assembling concentrations were determined which shows that TrcB has more affinity to self-assemble compared to TrcC. In membrane-mimetic environment like in trifluoroethanol, both the peptides tend to self-assemble more strongly than in aqueous media but SDS micelles cause disruption of higher order structures and disintegrate them into their monomeric forms. The divalent metal ion Ca\(^{2+}\) acts as a chaotropic agent to disrupt the peptide aggregates, especially, the disruption is more effective in aqueous media. Thus it can be suggested that if these peptides needs to be used as drugs in their monomeric form then encapsulation within micelles are best suited which will facilitate drug delivery.

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