Protein Expression and Purification 102 (2014) 63–68

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Expression, purification, and micelle reconstitution of antimicrobial piscidin 1 and piscidin 3 for NMR studies

Wen Chen [a], Myriam L. Cotten [b,*]

[a] Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, United States
[b] Department of Chemistry, Hamilton College, 198 College Hill Road, Clinton, NY 13323, United States

ARTICLE INFO

Article history:
Received 22 July 2014
and in revised form 31 July 2014
Available online 13 August 2014

Keywords:
Amphipathic antimicrobial peptides
$^{13}C/^{15}N$ uniformly labeled
Piscidin
Membrane-active peptides
NMR
Micelles

ABSTRACT

Piscidin 1 and piscidin 3, which were discovered in the mast cells of hybrid striped sea bass, are homologous antimicrobial peptides that are active against drug-resistant bacteria. Piscidin 1, the more antimicrobial and hemolytic peptide, also has anti-HIV-1 and anti-cancer properties. To understand the reasons underlying the different biological activities of the two peptides and identify principles to design antimicrobial drugs with improved efficacy and lower toxicity, their atomic-level structures must be obtained under physiologically-relevant conditions. High-resolution backbone structures of both piscidins exist in the presence of hydrated phospholipid bilayers but full structures that include the side chains are missing. Here, the piscidins 1 and 3 genes were cloned into the TrpLE vector. The corresponding TrpLE-piscidin fusion partners were expressed in *Escherichia coli* and recovered from inclusion bodies. Following steps that included Ni–NTA chromatography, cyanogen bromide cleavage of the fusion proteins, and reverse-phase HPLC, purified piscidins 1 and 3 were recovered in very good yield and characterized by NMR. High quality ^{15}N-^{1}H HSQC spectra of piscidins 1 and 3 bound to SDS micelles were collected, demonstrating the feasibility of producing and purifying the isotopically-labeled piscidin peptides required to determine their full structures by multidimensional NMR spectroscopy.

Introduction

Antimicrobial peptides (AMPs[1]) play an essential role in the innate immune system [1–4]. Due to their activity against drug-resistant bacteria and low induction of bacterial resistance, they have been the focus of intense research aimed at producing novel antimicrobial drugs. While their direct antimicrobial effects are generally due to disruption of the target's cell membranes, their mechanism of action remains a robustly debated topic [2,5–8]. In the presence of bacterial cell mimics, AMPs generally adopt amphipathic secondary structures that segregate polar and non-polar residues [9,10]. While amphipathicity is a major consideration in the design of therapeutically useful AMPs [11–17], it is also recognized that amphipathically imperfect (rather than perfect) peptides may be more effective at disrupting membranes [8]. To further improve the rational design of AMPs that are highly specific to bacterial membranes and nontoxic to host cells, molecular determinants conducive to disruption of microbial membranes have to be obtained through detailed structural characterization of efficacious AMPs under native-like conditions. NMR has emerged as the method of choice to solve structures of amphipathic peptides bound to lipid membranes or lipid-mimetic systems. In the antimicrobial database (APD) [18], only 13% of the AMPs have known 3D structures, 90% of which were elucidated by NMR.

Piscidins constitute a large family of AMPs that are highly efficacious against Gram-positive and Gram-negative bacteria [19–22]. Members of the piscidin family have strongly conserved amino ends and contain more histidines than on average in the APD [18]. Piscidin 1 (FFHHIFRGIVHVGKTIHRLVTG), which has been widely investigated, is classified as one of the top six AMPs in terms of its potency against a broad spectrum of bacteria and HIV-1 [23,24]. While the mechanism of action for HIV-inhibition is not known, it has been suggested that the presence of cationic arginine side chains is important [24]. In addition, piscidin 1 has anti-cancer properties against HeLa and HT1080 cells, leading to apoptosis and necrosis in HT1080 cells [25]. Piscidin 2, which is identical to piscidin 1 to the exception of a K to R substitution at residue 18, has similar antimicrobial effectiveness [19]. Piscidin 3 (FIHHIFRGIVHAGRSIGRFLTG), which is homologous to both piscidins 1 and 2, is significantly less antimicrobial and hemolytic for reasons that are not yet understood [19,26]. Biophysical studies of piscidins 1

* Corresponding author. Tel.: +1 315 859 4243.
E-mail address: mcotten@hamilton.edu (M.L. Cotten).

[1] *Abbreviations used:* AMP, antimicrobial peptide; APD, antimicrobial peptide data base; DPC, dodecylphosphocholine; IPTG, isopropyl-thio-galactoside; RP-HPLC, reverse phase high performance liquid chromatography; SDS, sodium dodecyl sulfate; HSQC, heteronuclear single quantum coherence.

http://dx.doi.org/10.1016/j.pep.2014.08.001
1046-5928/

and 3 could help address this question and inform the design of a modified piscidin that is as antimicrobial as piscidin 1 and as non-toxic as piscidin 3.

Previously, solid-state NMR studies in our lab resulted in the high-resolution backbone structures of piscidins 1 and 3 in the presence of Gram-positive and Gram-negative bacterial cell membrane mimics [26–31]. The peptides were found to be more α-helical and deeply inserted in the Gram-positive membrane mimic, consistent with stronger antimicrobial effects on these bacteria. These studies also revealed that both piscidins 1 and 3 are kinked at a conserved central glycine, which allows the peptides to optimize their interactions with the hydrophobic part of the bilayer. Since the studies were limited to using backbone-labeled peptides, it was not possible to address the structural features of the side chains even though they mediate important peptide-lipid interactions and are very likely involved in allowing the peptide to convert from a surface-bound state to an inserted state from which pore formation and/or bilayer disruption ensues. Structures of piscidin 1 exist in the presence of sodium dodecyl sulfate (SDS) and dodecylphosphocholine (DPC) micelles but they differ significantly [32,33]. This underscores the importance of testing different lipid systems and finding conditions that best reproduce native-like conditions. No full structures of piscidin 3 have been obtained even though this would help better understand structure–function relationships in the piscidin family. Uniformly $^{13}C/^{15}N$ labeled piscidin is a prerequisite to efficiently determining full NMR structures in the presence of micelles, bicelles, or hydrated lipid bilayers. Such peptides could be chemically synthesized using uniformly labeled amino acids but this approach could be onerous and result in low yields. Here, we describe a biosynthetic method to produce and purify piscidins 1 and 3 in very good yields. NMR spectra of the two peptides reconstituted in SDS micelles demonstrate the high quality of the synthesis and purification process. This approach can be used to produce within a few days not only piscidins 1 and 3 but also analogs of enhanced therapeutic effects.

Materials and methods

Construction of piscidin expression vector

Codon-optimized DNA sequences of piscidin 1 and piscidin 3 were synthesized by GenScript company (New Jersey, USA). During synthesis, HindIII and BamHI sites were added at the respective N- and C-termini of the piscidin 1 and piscidin 3 genes. These genes were then cloned into the TrpLE vector [34], which provides kanamycin resistance. The cloning products of TrpLE-piscidin 1 and TrpLE-piscidin 3 were confirmed by DNA sequencing analysis. The resulting plasmids encoded an N-terminally 9X-histidine-tagged fusion protein of the 13 kDa TrpLE leader sequence and 2.5 kDa piscidin. The TrpLE sequence, which is derived from the *Escherichia coli* tryptophan operon leader region, directed the expression of proteins to inclusion bodies. Thanks to its His-Tag, the TrpLE-piscidin fusion protein could be separated from other impurities. The site of cleavage by cyanogen bromide was the single methionine residue at the carboxy-terminal of TrpLE. Upon cleavage with cyanogen bromide, the piscidin 1 and piscidin 3 sequences were released intact and separated from impurities using RP-HPLC.

Expression and purification of piscidin

The kanamycin-resistant TrpLE-piscidin plasmids were transformed into *E. coli* BL21 (DE3) cells. A single colony was taken from the plate and used to inoculate a 200 mL-culture in LB broth (Sigma) at 37 °C. The culture was grown overnight with shaking at 220 rpm before being used to start a large scale (4 L) expression of natural abundance proteins at 37 °C. After the OD_{600}, which started at 0.1, reached 0.5, the cells were subjected to cold shock treatment by placing them on ice for 30 min. This treatment allowed us to cool down the culture that was at 37 °C and improve the yield of the expression, which was carried out at 21 °C as explained below. Isopropyl-thio-galactoside (IPTG, Sigma) was added to the cooled culture to a final concentration of 0.2 mM, as needed to initiate expression. Optimal expression was achieved when the culture was shaken at 220 rpm and 21 °C for 24 h. Cell pellets were collected by centrifugation at 4000*g* and 4 °C for 30 min, and stored at -80 °C until protein purification was carried out. ^{15}N- and/or ^{13}C-labeled peptides were produced from M9-based minimal medium containing $^{15}NH_4Cl$ (1 g/L, Cambridge Isotope Laboratory, MA) and/or ^{13}C glucose (3 g/L, Cambridge Isotope Laboratory, MA) instead of the LB broth used for the large expression cultures.

For purification, cells were resuspended in the lysis buffer (100 mM Tris–HCl, 200 mM NaCl, pH = 8.0) at a ratio was 5 mL of lysis buffer for 1 g of cell pellets. Cell lysis was achieved on ice using a sonicator set at 45% power over 10 cycles, each alternating 20 s on and 30 s off. Sonication was carried out twice for each sample. To separate the soluble fraction from the inclusion bodies, the cell lysates were centrifuged for 30 min at 37,500*g* and 4 °C. The inclusion-body fraction was then extracted using breaking buffer (6 M guanidine HCl, 50 mM Tris, 200 mM NaCl, 1% Triton X-100, pH 8.0) and overnight rocking on a Nutator. Alternatively, a glass homogenizer was used to crush inclusion bodies into breaking buffer. The guanidine-dissolved cell pellets were centrifuged for 30 min at 37,500*g* and 4 °C, and the supernatant was collected before being incubated with an Ni–NTA resin (Thermo Scientific) (1 g of pellet per 1 mL of resin) that had been pre-equilibrated for 30 min with 5 column volumes of breaking buffer. The mixture was then transferred to a gravity-flow column (Bio-rad). The resin was washed with 5 column volumes of 8 M urea solution, followed by 5 column volumes of water. The resin was then eluted with 2 column volumes of formic acid. Fractions from the flow-through, wash, and elution of each step were collected and identified by SDS–PAGE. Fractions that contained the fusion protein were diluted to 80% formic acid/20% water (v/v) in preparation for cyanogen bromide cleavage and cleaved using 2 g of cyanogen bromide for every 10 mL of 80% formic acid/20% water (v/v) eluate.

The fractions from the cleavage were combined and placed into dialysis cassettes (2000 MWCO) (Thermo Scientific) and dialyzed twice in water for about one hour each. After dialysis and analysis by SDS–PAGE, the fractions containing piscidin were lyophilized and dissolved in a small volume (about 10 mL) of 50% formic acid/50% water (v/v). This solution was filtered before being purified by RP-HPLC on a ZORBAX SB-C18 column (Agilent). A linear gradient of 0–100% of buffer B (90% acetonitrile, 0.1% trifluoroacetic acid) over buffer A (10% acetonitrile, 0.1% trifluoroacetic acid) was initiated after the first peak at a flow rate of 3 mL/min for 60 min. The identity and purity of piscidin 1 and piscidin 3 were confirmed by mass spectrometry (4800 Plus MALDI Analyzer). Fractions containing pure piscidin peptides were lyophilized and stored at −20 °C until further use.

Reconstitution of piscidin into SDS micelles

^{15}N-labeled piscidin 1 and piscidin 3 peptides were directly dissolved in the following detergent buffer: 4% SDS (Sigma), 50 mM sodium phosphate, 100 mM sodium chloride, pH = 6.5.

HSQC of piscidin

After reconstitution of piscidin into SDS micelles, ^{15}N-^{1}H HSQC spectra of each sample were acquired at 313 K over two hours using a Bruker 600 MHz spectrometer fitted with a cryogenic

probe. We used 313 K because higher temperatures are usually favorable to solution NMR experiments on membrane proteins and peptides. For each time increment, 16 transients were taken with 4096 and 256 points in the ^{15}N and ^{1}H dimensions, respectively. Spectra were collected with respective width and center of 12 and 4.7 ppm for proton, and 26 and 117 ppm for nitrogen.

Results

Overexpression and purification of piscidin

The expression protocol of recombinant piscidin is summarized in Fig. 1A and explained in the Materials and Methods section. The highest protein expression level of the TrpLE-piscidin fusion protein was obtained when induction was done with 0.2 mM IPTG and bacteria grew for 24 h at 21 °C (Fig. 1B). A clear difference is observed on the SDS–PAGE gel between induced and uninduced cells. TrpLE-piscidin fusion protein (15 kDa) was observed after IPTG induction. Protein of this size could not be detected if IPTG was not added.

The purification procedure is also outlined in Fig. 1A. Sonication was used to lyse the cells and the fusion protein was recovered from the inclusion bodies, which required the use of 6 M guanidine-HCl. The guanidine-dissolved fusion protein was incubated with Ni–NTA resin before washing with urea and water. Most of the impurities were washed away before the TrpLE-piscidin fusion protein was eluted using 100% formic acid. After elution from the Ni–NTA column, the TrpLE-piscidin fusion protein was detected as the strongest band on the SDS–PAGE gel (>90% purity) (Fig. 2, lane 3). At least 80% of the fusion protein was cleaved after incubation with cyanogen bromide for 2 h (Fig. 2, lanes 4 and 5).

The cleavage product was then lyophilized and dissolved in 50% formic acid/50% water (v/v), followed by RP-HPLC purification using a semi-preparative C18 column. During the 10–90% acetonitrile gradient, piscidin eluted at about 30% acetonitrile (Fig. 3A). The identity of piscidin was confirmed by mass spectrometry (Fig. 3B). As exemplified for ^{15}N-uniformly labeled piscidin 3 in Fig. 3, the well-defined peak isolated from HPLC and the strong agreement between the measured (2528.49 Da) and theoretical (2528.93 Da) masses show that the expression and purification strategy used for piscidin was successful. Pure piscidin was then freeze-dried and stored at −20 °C until further use. The final yield of expressing and purifying isotopically-labeled piscidin was about

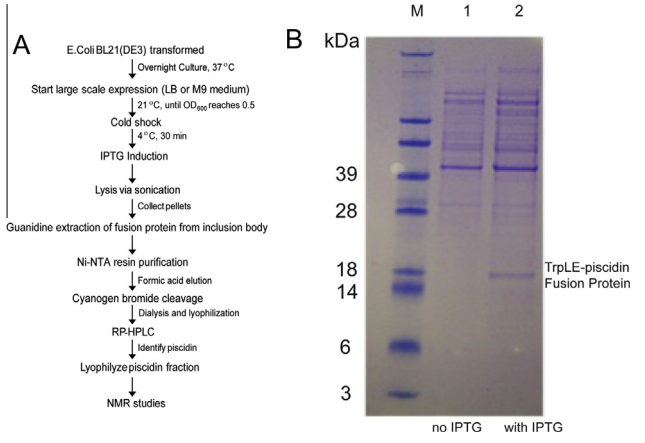

Fig. 1. Procedure for the expression and purification of recombinant piscidin. (A) Flowchart for the expression and purification of piscidin. (B) SDS–PAGE of the samples pertaining to the expression of piscidin 3 before and after induction with IPTG. Lane M, molecular weight markers; lane 1, whole cell lysate uninduced; lane 2, cell lysate induced with 0.2 mM IPTG. Similar results were obtained for the expression of piscidin 1.

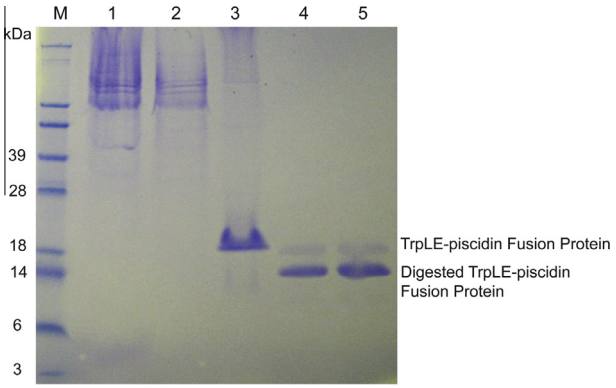

Fig. 2. SDS–PAGE of Ni–NTA resin purification and cyanogen bromide cleavage of the TrpLE-piscidin 3 fusion protein. Lane 1, flow-through of the lysate after incubation with Ni–NTA resin; lane 2, urea wash of Ni–NTA resin; lane 3, relatively pure TrpLE-piscidin fusion protein after formic acid elution; lane 4, 1 h after cyanogen bromide cleavage; lane 5, 2 h after cyanogen bromide cleavage. The expression of piscidin 1 yielded similar results.

1 mg of purified peptide per liter of $^{15}N/^{13}C$-labeled M9 medium and twice as much in the LB medium. Similar results were obtained for piscidin 1 and piscidin 3.

HSQC of piscidin in micelles

Detergent micelles represent the environment most commonly chosen to investigate membrane proteins and peptides in solution. Here, HSQC spectra were obtained for piscidin 1 and piscidin 3 in SDS micelles at pH 6.5. Both spectra demonstrate excellent resolution and separation (Fig. 4), consistent with the finding by CD as well as solution and solid-state NMR that both 22-mers adopt well defined structures in SDS micelles and lipid bilayers [26,27,32]. Backbone amide protons for the first one or two residue(s) of a polypeptide may not give rise to any signal in HSQC spectra because of the fast exchange with the aqueous solvent at pH 6.5. Accordingly, 20 amide backbone cross peaks with proton frequencies between 7.5 and 9 ppm are observed for piscidin 3. The three peaks that resonate around 7 ppm in proton frequency are assigned the three folded arginine side chains [35]. For piscidin 1, 18 amide backbone cross peaks are detected probably due to peak overlaps in addition to solvent exchange. Obvious chemical shift differences exist between piscidins 1 and 3 as expected if the two peptides are conformationally different in SDS micelles.

Discussion

Piscidins 1 and 3 are good archetypes of AMPs, and therefore can be used as templates to design new antimicrobial drugs. While high-resolution backbone structures of piscidins 1 and 3 bound to lipid bilayers exist [26], there are no full structures in this native-like environment. NMR sample preparation is the first step for obtaining these structures. Due to its biological activity against microbes, piscidin is difficult to express in bacteria. Moon et al. reported multiple challenges when they used a ubiquitin construct to express piscidin 1 [35]. Here, we used TrpLE as the expression partner and expressed both piscidins 1 and 3. This directs expressed protein to inclusion bodies. Since the cleavage is done with cyanogen bromide instead of an enzyme, no refolding step, which could result in lower yields, is needed. Overall, this approach results in very good expression level and yield, and a process that can be completed within a few days. Overall, one liter of M9 produces enough ^{15}N-labeled peptide to make multiple 0.4 mM-samples for solution NMR while 6 L yield enough $^{13}C/^{15}N$-labeled peptide for oriented sample solid-state NMR.

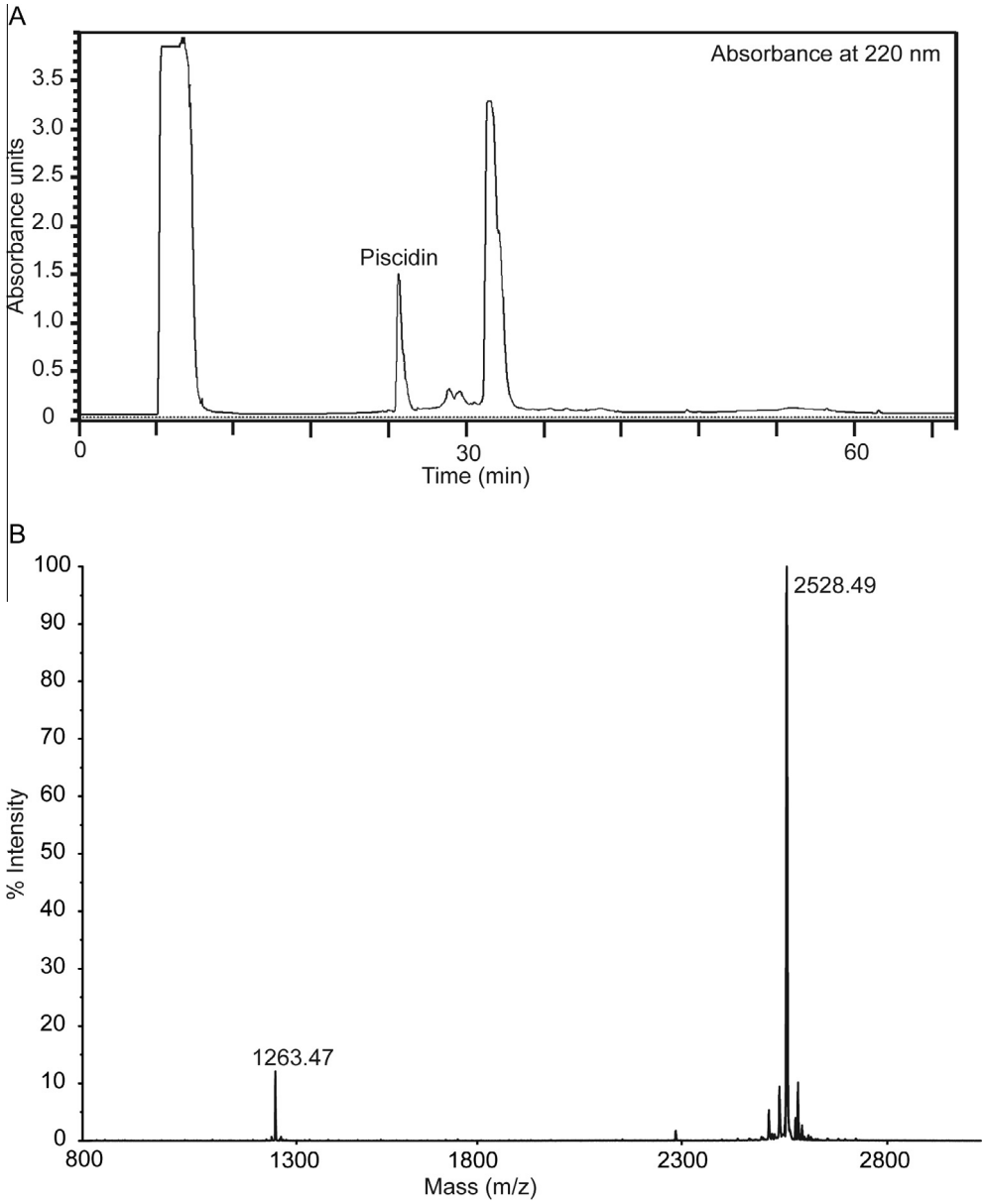

Fig. 3. HPLC purification and mass spectrometry identification of piscidin 3. (A) HPLC chromatogram of cyanogen bromide digest mix, monitored at 220 nm. Single isolated piscidin 3 peak appears around 30% acetonitrile. (B) Mass chromatogram of ^{15}N labeled piscidin 3. Theoretical mass is 2528.93 Da while the measured mass is 2528.49 Da.

Ni–NTA resins are effective to purify polyhistidine-tagged recombinant proteins under both native [36–39] and denaturing conditions [40]. By altering the number of histidines in the tag, the elution profile and separation of proteins from each other and impurities can be improved. Formic acid can replace imidazole to displace strong binders. Here, following formic acid elution, the TrpLE-piscidin fusion protein could readily be cleaved with cyanogen bromide.

The high efficiency of cyanogen bromide cleavage at the C-end of methionine residues is advantageous to generate methionine-free polypeptides. Here, piscidin was released from the TrpLE-piscidin partner thanks to a methionine at the C-end of the TrpLE protein. Because the cleavage is carried out in formic acid, it could result in a formylated peptide product [41]. Therefore, the reaction is typically executed within a few hours, and under an inert gas (e.g. nitrogen, argon). Dissolving the peptide in formic acid before adding the cyanogen bromide produces acidic and reducing conditions that help prevent the oxidation of the side chains [42]. If a formylated peptide had been present or the amino side chains had been oxidized, the change in charge state and hydrophobicity of the peptide would have affected its elution time by HPLC. Since a well-defined HPLC peak was obtained, the mass spectrum identified a peptide of correct molecular weight, and the NMR spectrum is consistent with a unique piscidin species, it is concluded that an intact peptide of high purity was collected by HPLC and used for the NMR study. Relatively high concentration of cyanogen bromide (20% w/v) in the 80% formic acid/20% water solution helped increase the cleavage yield for the TrpLE-piscidin fusion partner. Under these conditions, a two-hour reaction time translated into a yield greater than 90%.

We used an acetonitrile gradient on a C18 reverse phase HPLC column to separate piscidin from the cleavage mixture. However, due to the peptide tendency to aggregate and precipitate at high concentration, multiple injections were required to recover enough highly purified peptide to prepare NMR samples yielding high signal-to-noise ratio spectra. As an alternative, hydrophilic-interac-

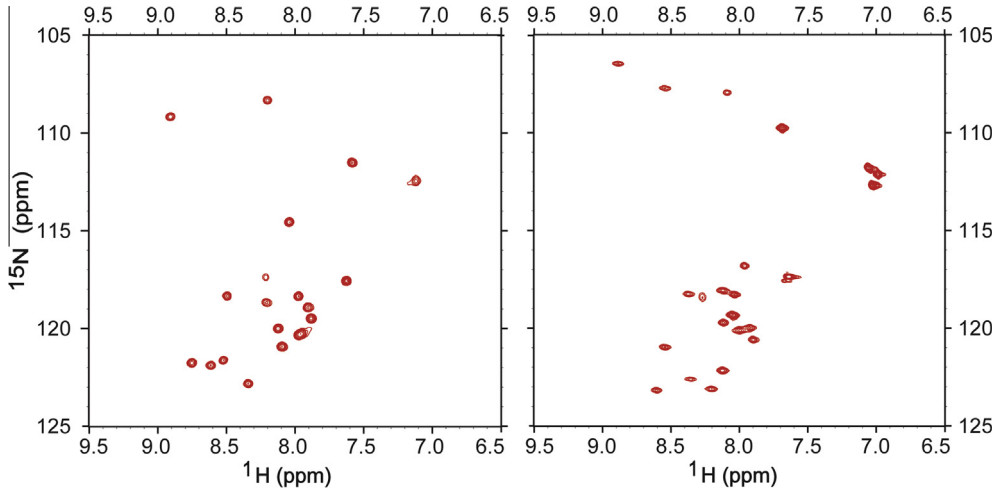

Fig. 4. ^{15}N-^{1}H HSQC of piscidin 1 (left) and piscidin 3 (right) in 4% SDS at pH 6.5. The peptide concentration was approximately 0.2 mM. The NMR samples also contained 50 mM sodium phosphate buffer and 100 mM NaCl in 10% D_2O. Piscidin has 22 residues and solvent exchange is expected to prevent the detection of signals for the first one or two residues at the amino end. Accordingly, 20 separate backbone amide cross peaks are observed for piscidin 3. For piscidin 1,18 separate cross peaks appear, probably due to overlapping peaks in addition to solvent exchange. Arginine side chains have proton signals around 7 ppm. Obvious spectral differences can be identified between piscidins 1 and 3, suggesting local conformation differences between them.

tion chromatography (HILIC) could be considered to purify piscidin. In this case, solutes with higher hydrophilicity will elute last [43]. Therefore, this may help prevent the precipitation of hydrophobic proteins and premature loss of column performance. Cell-free expression could also be attempted to synthesize and transfer piscidin into detergent micelles or aggregates that could then be purified [44].

To obtain high-quality NMR spectra of piscidin in detergents that mimic the amphipathic character of lipids, it is important to screen different buffer conditions and lipid mimics. Previous studies show that well resolved NMR spectra of membrane polypeptides can be obtained using various detergents, including DPC, 1,2-dicaproyl-1-sn-glycero-3-[phospho-RAC-(1-glycerol)] (DHPC), *N*,*N*-dimethyldodecylamine N-oxide (LDAO), 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LMPG), 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG), and SDS [39,45–48]. Anionic SDS micelles, which were previously used to investigate piscidin 1 and induced high helical content (88% based on CD)[32], were first tested here for piscidin 3. Bound to 25% anionic lipid bilayers made of 3:1 1-palmitoyl-2-oleoyl-sn-glycero-phosphatidylcholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-phosphoglycerol (POPG), piscidins 1 and 3 studied by CD and solid-state NMR were aligned parallel to the membrane surface and more than 90% helical [26]. While piscidin 1 also interacts with zwitterionic DPC micelles and becomes helical as shown by broader linewidths in ^{1}H NMR spectra and upfield shifts of the α-proton resonances, the helical content (65% based on CD) is lower than in the presence of anionic SDS micelles and 3:1 PC/PG bilayers [33]. As shown in Fig. 4, excellent HSQC spectra were obtained for both piscidins 1 and 3 bound to SDS micelles confirming that the micelles stabilize the secondary structure observed in the presence of lipid bilayers [26]. In the future, we also plan to reconstitute piscidin in isotropic and anisotropic bicelles as well as extended lipid bilayers. Piscidin could also be tested in the presence of zwitterionic or non-ionic detergents (e.g. beta-dodecylmaltoside) that are stabilizing to membrane proteins [49,50] but their nonionic character may reduce their interactions with the peptide, which is cationic and relies on electrostatic interactions in addition to hydrophobic interactions to bind micelles.

In conclusion, an effective and reliable method has been established to express and purify both unlabeled and isotopically-labeled piscidins 1 and 3. Furthermore, the peptides were successfully reconstituted into detergent micelles to mimic the amphipathic nature of lipids. The protocol could be used to produce piscidin analogs with enhanced therapeutics properties, in particular lower hemolycity than piscidin 1. Overall, it provides a strong foundation for future structure–function relationship studies in piscidin analogs by NMR, as needed to better understand piscidin's role as an antimicrobial and anticancer peptide.

Acknowledgments

This research was supported by a Grant from the National Science Foundation (CHE-0832571). The authors acknowledge Professor Chunyu Wang from Rensselaer Polytechnic Institute for helpful advice and discussion in the early stages of the project.

References

[1] E.F. Haney, R.E.W. Hancock, Peptide design for antimicrobial and immunomodulatory applications, Biopolymers 6 (2013) 572–583.
[2] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria, Nat. Rev. Microbiol. 3 (2005) 239–250.
[3] H.G. Boman, Antibacterial peptides: basic facts and emerging concepts, J. Intern. Med. 254 (2003) 197–215.
[4] M. Zasloff, Antimicrobial peptides of multicellular organisms, Nature 415 (2002) 389–395.
[5] J. Hale, R.E.W. Hancock, Alternative mechanisms of action of cationic antimicrobial peptides on bacteria, Expert Rev. Anti-Infect. Ther. 5 (2007) 951–959.
[6] B. Bechinger, E.S. Salnikov, The membrane interactions of antimicrobial peptides revealed by solid-state NMR spectroscopy, Chem. Phys. Lipids 165 (2012) 282–301.
[7] R.M. Epand, H.J. Vogel, Diversity of antimicrobial peptides and their mechanisms of action, Biochim. Biophys. Acta 1462 (1999) 11–28.
[8] W.C. Wimley, Describing the mechanism of antimicrobial peptide action with the interfacial activity model, ACS Chem. Biol. 5 (2010) 905–917.
[9] A.J. Krauson, J. He, W.C. Wimley, Gain-of-function analogues of the pore-forming peptide melittin selected by orthogonal high-throughput screening, J. Am. Chem. Soc. 134 (2012) 12732–12741.
[10] N. Uematsu, K. Matsuzaki, Polar angle as a determinant of amphipathic α-helix–lipid interactions: a model peptide study, Biophys. J. 79 (2000) 2075–2083.
[11] E.F. Haney, R.E.W. Hancock, Peptide design for antimicrobial and immunomodulatory applications, Biopolymers 6 (2013) 572–583.
[12] J.P. Powers, R.E. Hancock, The relationship between peptide structure and antibacterial activity, Peptides 24 (2003) 1681–1691.
[13] L.M. Yin, M.A. Edwards, J. Li, C.M. Yip, C.M. Deber, Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide–membrane interactions, J. Biol. Chem. 287 (2012) 7738–7745.

[14] E. Glukhov, M. Stark, L.L. Burrows, C.M. Deber, Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes, J. Biol. Chem. 280 (2005) 33960–33967.
[15] M. Dathe, T. Wieprecht, Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells, Biochim. Biophys. Acta 1462 (1999) 71–87.
[16] C.D. Fjell, J.A. Hiss, R.E.W. Hancock, G. Schneider, Designing antimicrobial peptides: form follows function, Nat. Rev. Drug Discov. 11 (2012) 37–51.
[17] Y. Chen, M.T. Guarnieri, A.I. Vasil, M.L. Vasil, C.T. Mant, R.S. Hodges, Role of peptide hydrophobicity in the mechanism of action of alpha-helical antimicrobial peptides, Antimicrob. Agents Chemother. 51 (2007) 1398–1406.
[18] G. Wang, X. Li, Z. Wang, APD2: the updated antimicrobial peptide database and its application in peptide design, Nucleic Acids Res. 37 (2009) D933–D937.
[19] U. Silphaduang, E. Noga, Antimicrobials: peptide antibiotics in mast cells of fish, Nature 414 (2001) 268–269.
[20] X. Lauth, H. Shike, J.C. Burns, M.E. Westerman, V.E. Ostland, J.M. Carlberg, J.C.V. Olst, V. Nizet, S.W. Taylor, C. Shimizu, P. Bulet, Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass, J. Biol. Chem. 277 (2002) 5030–5039.
[21] J. Menousek, B. Mishra, M.L. Hanke, C.E. Heim, T. Kielian, G.S. Wang, Database screening and in vivo efficacy of antimicrobial peptides against methicillin-resistant *Staphylococcus aureus* USA300, Int. J. Antimicrob. Agents 39 (2012) 402–406.
[22] W.S. Sung, J.H. Lee, D.G. Lee, Fungicidal effect of piscidin on *Candida albicans*: pore formation in lipid vesicles and activity in fungal membranes, Biol. Pharm. Bull. 31 (2008) 1906–1910.
[23] G. Wang, Database-guided discovery of potent peptides to combat HIV-1 or superbugs, Pharmaceuticals 6 (2013) 728–758.
[24] G. Wang, K.M. Watson, A. Peterkofsky, R.W. Buckheit Jr., Identification of novel human immunodeficiency virus type 1-inhibitory peptides based on the antimicrobial peptide database, Antimicrob. Agents Chemother. 54 (2010) 1343–1346.
[25] H.-J. Lin, T.-C. Huang, S. Muthusamy, J.-F. Lee, Y.-F. Duann, C.-H. Lin, Piscidin-1, an antimicrobial peptide from fish (hybrid striped bass *Morone saxatilis* × *M. chrysops*), induces apoptotic and necrotic activity in HT1080 cells, Zool. Sci. 29 (2012) 327–332.
[26] B.S. Perrin Jr., Y. Tian, R. Fu, C.V. Grant, E.Y. Chekmenev, W.E. Wieczorek, A.E. Dao, R.M. Hayden, C.M. Burzynski, R.M. Venable, M. Sharma, S.J. Opella, R.W. Pastor, M.L. Cotten, High-resolution structures and orientations of antimicrobial peptides piscidin 1 and piscidin 3 in fluid bilayers reveal tilting, kinking, and bilayer immersion, J. Am. Chem. Soc. 136 (2014) 3491–3504.
[27] E.Y. Chekmenev, B.S. Vollmar, K.T. Forseth, M.N. Manion, S.M. Jones, T.J. Wagner, R.M. Endicott, B.P. Kyriss, L.M. Homem, M. Pate, J. He, J. Raines, P.L. Gor'kov, W.W. Brey, D.J. Mitchell, A.J. Auman, M. Ellard-Ivey, J. Blazyk, M. Cotten, Investigating molecular recognition and biological function at interfaces using piscidins, antimicrobial peptides from fish, Biochim. Biophys. Acta 1758 (2006) 1359–1372.
[28] E. Chekmenev, B. Vollmar, M. Cotten, Can antimicrobial peptides scavenge around a cell in less than a second?, Biochim Biophys. Acta 1798 (2010) 228–234.
[29] E.Y. Chekmenev, S.M. Jones, Y.N. Nikolayeva, B.S. Vollmar, T.J. Wagner, P.L. Gor'kov, W.W. Brey, M.N. Manion, K.C. Daugherty, M. Cotten, High-field NMR studies of molecular recognition and structure–function relationships in antimicrobial piscidins at the water–lipid bilayer interface, J. Am. Chem. Soc. 128 (2006) 5308–5309.
[30] R. Fu, E.D. Gordon, D.J. Hibbard, M. Cotten, High resolution heteronuclear correlation NMR spectroscopy of an antimicrobial peptide in aligned bilayers at high magnetic field: peptide–water interactions at the water–bilayer interface, J. Am. Chem. Soc. 131 (2009) 10830–10831.
[31] Anna A. De Angelis, Christopher V. Grant, Matthew K. Baxter, Jason A. McGavin, Stanley J. Opella, Myriam L. Cotten, Amphipathic antimicrobial piscidin in magnetically aligned lipid bilayers, Biophys. J. 101 (2011) 1086–1094.
[32] S.A. Lee, Y.K. Kim, S.S. Lim, W.L. Zhu, H. Ko, S.Y. Shin, K. Hahm, Y. Kim, Solution structure and cell selectivity of piscidin 1 and its analogues, Biochemistry 46 (2007) 3653–3663.
[33] S. Campagna, N. Saint, G. Molle, A. Aumelas, Structure and mechanism of action of the antimicrobial peptide piscidin, Biochemistry 46 (2007) 1771–1778.
[34] J.P. Staley, P.S. Kim, Formation of a native-like subdomain in a partially folded intermediate of bovine pancreatic trypsin inhibitor, Protein Sci. 3 (1994) 1822–1832.
[35] W.J. Moon, D.K. Hwang, E.J. Park, Y.M. Kim, Y.K. Chae, Recombinant expression, isotope labeling, refolding, and purification of an antimicrobial peptide, piscidin, Protein Expr. Purif. 51 (2007) 141–146.
[36] W. Chen, L. Li, Z. Du, J. Liu, J.N. Reitter, K.V. Mills, R.J. Linhardt, C. Wang, Intramolecular disulfide bond between catalytic cysteines in an intein precursor, J. Am. Chem. Soc. 134 (2012) 2500–2503.
[37] W. Chen, E. Gamache, D. Richardson, Z. Du, C. Wang, Expression, purification, and reconstitution of the transmembrane domain of the human amyloid precursor protein for NMR studies, Protein Expr. Purif. 81 (2012) 11–17.
[38] Z. Du, J. Liu, C.D. Albracht, A. Hsu, W. Chen, M.D. Marieni, K.M. Colelli, J.E. Williams, J.N. Reitter, K.V. Mills, C. Wang, Structural and mutational studies of a hyperthermophilic intein from DNA polymerase II of *Pyrococcus abyssi*, J. Biol. Chem. 286 (2011) 38638–38648.
[39] W. Chen, E. Gamache, D.J. Rosenman, J. Xie, M.M. Lopez, Y.M. Li, C. Wang, Familial Alzheimer's mutations within APPTM increase Abeta42 production by enhancing accessibility of epsilon-cleavage site, Nat. Commun. 5 (2014) 3037.
[40] M.E. Call, J.R. Schnell, C. Xu, R.A. Lutz, J.J. Chou, K.W. Wucherpfennig, The structure of the zetazeta transmembrane dimer reveals features essential for its assembly with the T cell receptor, Cell 127 (2006) 355–368.
[41] H.S. Duewel, J.F. Honek, CNBr/formic acid reactions of methionine- and trifluoromethionine-containing lambda lysozyme: probing chemical and positional reactivity and formylation side reactions by mass spectrometry, J. Protein Chem. 17 (1998) 337–350.
[42] R. Kaiser, L. Metzka, Enhancement of cyanogen bromide cleavage yields for methionyl-serine and methionyl-threonine peptide bonds, Anal. Biochem. 266 (1999) 1–8.
[43] A.J. Alpert, Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds, J. Chromatogr. 499 (1990) 177–196.
[44] B. Schneider, F. Junge, V.A. Shirokov, F. Durst, D. Schwarz, V. Dotsch, F. Bernhard, Membrane protein expression in cell-free systems, Methods Mol. Biol. 601 (2010) 165–186.
[45] L. Columbus, J. Lipfert, H. Klock, I. Millett, S. Doniach, S.A. Lesley, Expression, purification, and characterization of *Thermotoga maritima* membrane proteins for structure determination, Protein Sci. 15 (2006) 961–975.
[46] R.D. Krueger-Koplin, P.L. Sorgen, S.T. Krueger-Koplin, I.O. Rivera-Torres, S.M. Cahill, D.B. Hicks, L. Grinius, T.A. Krulwich, M.E. Girvin, An evaluation of detergents for NMR structural studies of membrane proteins, J. Biomol. NMR 28 (2004) 43–57.
[47] R.C. Page, J.D. Moore, H.B. Nguyen, M. Sharma, R. Chase, F.P. Gao, C.K. Mobley, C.R. Sanders, L. Ma, F.D. Sonnichsen, S. Lee, S.C. Howell, S.J. Opella, T.A. Cross, Comprehensive evaluation of solution nuclear magnetic resonance spectroscopy sample preparation for helical integral membrane proteins, J. Struct. Funct. Genomics 7 (2006) 51–64.
[48] S.F. Poget, M.E. Girvin, Solution NMR of membrane proteins in bilayer mimics: small is beautiful, but sometimes bigger is better, Biochim. Biophys. Acta 1768 (2007) 3098–3106.
[49] A.M. Seddon, P. Curnow, P.J. Booth, Membrane proteins, lipids and detergents: not just a soap opera, Biochim. Biophys. Acta Biomembr. 1666 (2004) 105–117.
[50] L. Columbus, J. Lipfert, K. Jambunathan, D.A. Fox, A.Y. Sim, S. Doniach, S.A. Lesley, Mixing and matching detergents for membrane protein NMR structure determination, J. Am. Chem. Soc. 131 (2009) 7320–7326.