

Chapter 2

Biological Active Antifungal Peptides

2.1 Introduction

The emergence of drug resistant fungal pathogens urgently calls for new generation of antifungal drugs with improved pharmacokinetics and pharmacodynamics. Antimicrobial peptides (AMPs) are mainly cationic and amphiphilic peptides composed of less than 50 amino acids, produced by diverse organisms for killing various kinds of invade bacteria, fungi, and viruses while have low cytotoxicity toward the organism themselves [1]. Due to their new mechanisms of microbicidal action and scarce of resistance, they have been recognized as a gold mine of antimicrobial drugs.

Defensin is a widely spread antimicrobial peptide within plants and animals; it possesses a compact 3 dimensional structure stabilized by several disulfide bridges [2]. Plant defensin, the innate guarding line against invaders, harbors antifungal activity, while showing limited toxicity to mammalian cell lines. Specifically, it was reported that defensins isolated from the white cloud beans possess both antifungal and antibacterial activities which even retained after trypsin treatment [3]. Although defensins are potential drug candidates, some obstacles need to be addressed: (1) the protein is difficult to separate; (2) the biosynthesis of defensins has low efficiency; and (3) in vivo activity and resistance still need to be improved. So, the semisynthesis of the biologically active form of the peptide holds promise.

While biologists put their efforts in solving crystal structures and biological activities of defensin proteins, more and more attention has been paid to the partial sequence of this protein family [4]. Several structural parameters, such as amphipathicity, hydrophobicity, hydrophobic momentum, alpha helicity, and the net positive charges, play an important role in antibacterial peptides' bioactivities [5]. Accordingly, partial sequence of the protein might still possess biological property

of the parental protein. So we decided to perform a structural-activity analysis of the whole sequence of defensin.

One of the plant defensin has been crystallized and was shown in Fig. 2.1 [6]. It is a macrocyclic protein containing three β sheets and one α helix, stabilized by four disulfide bonds. As total synthesis of defensin is not cost effective, we decided to fragment the protein into basic units based on the secondary structures. In the crystal structure, the first short β sheet is linked to a rigid α helix, and then to two adjacent β sheets (the yellow line represents the disulfide bond). And to simplify the structure, the 1st β sheet was deleted in the first round optimization, and then there were two optimizing strategies: one was to omit the 2nd β sheet to form a two disulfide bonded, α helix- β sheet domain (Peptide I) and the other one was to omit the α helix to form a β loop domain (Peptide II).

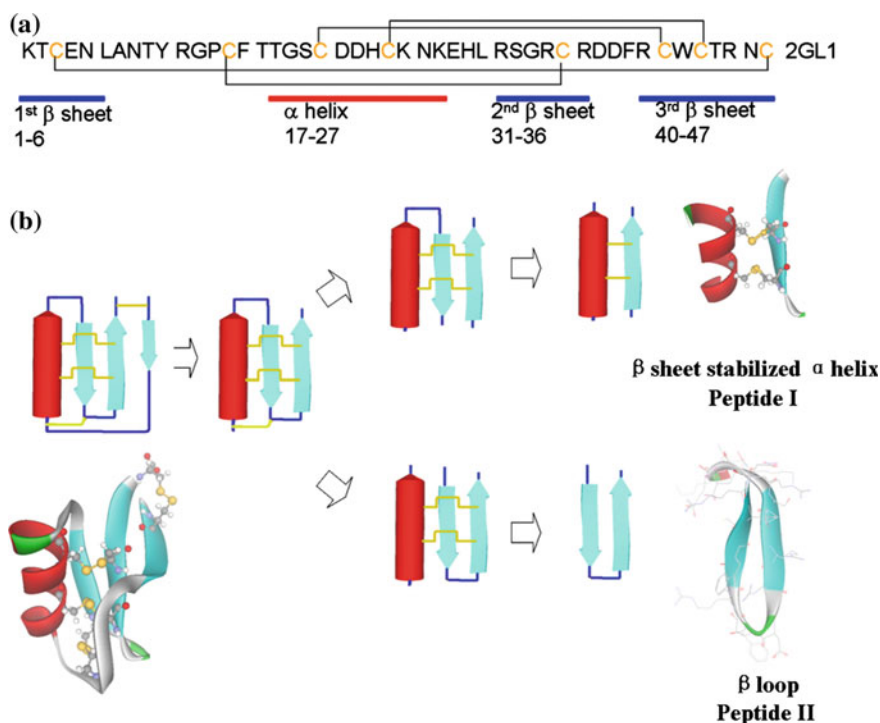


Fig. 2.1 Primary sequence and structure fragmentation of a plant defensin (PDB ID 2GL1). **a** Primary sequence of 2GL1. Cys are linked by disulfide bridges. Red line represents for the α helix and blue line represents for the β sheet. **b** 3-dimensional structure of 2GL1 and step by step structural fragmentation of the protein

2.2 Experimental Section

2.2.1 Peptide Synthesis and Purification

Peptides were manually synthesized based on standard Fmoc solid phase peptide synthesis protocol using HBTU/HOBt activation strategy. Briefly, Rink Amide-ChemMatrix[®] resins (PCAS BioMatrix, Canada) with 0.1 mmol amine group were utilized in each synthesis. A solution containing Fmoc-protected amino acid, HBTU, HOBt, and DIPEA (with a ratio of 1:1:1:2 and 5 fold excess) in 2 mL was added to the resin and stirred for 30 min at RT. After confirming the completion of the coupling reaction by Kaiser Test, the resins were washed with DMF and deprotected in 5 mL 20 % piperidine in DMF (v/v) to remove the Fmoc group to allow the coupling of the following amino acid. Specifically in this chapter, N α -Fmoc-S-acetaminomethyl-L-cysteine (Fmoc-Cys(Amc)-OH) and N α -Fmoc-S-trityl-L-cysteine (Fmoc-Cys(Trt)-OH) were used to incorporate Cys with different side chain protecting groups. After the completion of the entire sequence, the terminal Fmoc was removed and the resins were washed by DMF, iso-propanol and n-hexane, respectively, and the resins were put in high vacuum overnight.

To every 100 mg resins, 2 mL final cleavage cocktail containing EDT, TIS, phenol, H₂O, and TFA (with a ratio of 1:2:2:2:33, v/v/w/v/v) was added. The cleavage reaction was allowed to proceed for 2 h at RT under stirring. After the resins were removed through filtration, ice-cold diethyl ester was added to the supernatant dropwise to precipitate the peptides. A final volume of 700 μ L 50 % ACN in H₂O (v/v) was used to dissolve the peptide pellet. After being filtered through a 0.2 μ m filter, the peptide solution was injected to RP-HPLC (Shimadzu, DGU 20A5, Japan) equipped with a C18 column (Shimadzu, 250 L \times 4.6, Japan). 0.1 % TFA in H₂O (v/v) and 0.1 % TFA in ACN (v/v) were used as the mobile phase A and B, respectively. For all the analytical HPLC trials, the total flow rate was set to be 1 mL/min and the B concentration raised from 0 to 95 % over 16 min following a linear gradient. For the purification of peptides in a larger scale by semi-prep HPLC columns (Grace, 218TP510, USA), the total flow rate was set to be 3 mL/min and the concentration of B raised linearly from 0 to 45 % over 24 min. The peptide peaks were collected, lyophilized, and validated by MALDI-TOF mass spectrometry analysis (Bruker, autoflex TOF/TOF, USA). For the conjugated peptide or peptide mixture, the mass spec was confirmed by LC-ESI. All the lyophilized peptides were stored at -20 °C.

2.2.2 Intermolecular Disulfide Bond Formation

6.3 mg alpha helix peptide (H-RC(Acm)WCTRNA-NH₂, pep 1), together with 8.5 mg bis(5-nitro-2-pyridyl) disulfide (DTNP, 4 equiv.) were dissolved in 1 mL acetic acid/H₂O mixture (3:1, v/v), stirring at RT, at different time points, aliquots of

the reaction mixture were quenched by 0.1 % TFA containing acetonitrile and the reaction was monitored by analytical RP-HPLC. After the completion of the reaction, the mixture were neutralized, filtered through a 0.2 μm membrane and purified by semi-prep HPLC and characterized by MALDI-TOF. 3.5 mg thiol activated α helix peptide (pep 2) was dissolved in 1 mL citric/phosphate buffer (pH 6), bubbled with argon for 5 min, to the solution was added 600 μL citric/phosphate buffer containing 4.4 mg β sheet peptide (H-TSNC(Acm)DDHCKNK-NH₂, equal equiv. pep 3) in the presence of argon to immediately give a bright yellow solution. The reaction was completed within 5 min as monitored by HPLC.

For the second disulfide bond formation, 1.1 mg heterodimeric peptide was dissolved in 400 μL acetic acid/H₂O mixture (4:1, v/v), to the solution was added 100 μL acetic acid/H₂O mixture containing 1.4 mg I₂, the reaction was allowed to proceed at RT with stirring for 3 h and quenched by the addition of 310 μL H₂O. And excess I₂ was reduced by the addition of 400 μL 10 mM ascorbic acid to avoid further oxidation. The reaction was also monitored by HPLC and characterized with LC-MS.

2.2.3 Intramolecular Disulfide Bond Formation

5.5 mg peptide IV was dissolved in 1 mL NaHCO₃/Na₂CO₃ buffer (0.1 mM, pH 9.4) bubbled with air and shaking at RT for 8 h, and the reaction was monitored by HPLC, after the completion of the reaction, the peptide was purified and lyophilized.

2.2.4 Biological Activity Assay

The assay of the synthetic antifungal peptides for antifungal activity toward *Candida Albicans*, which is human pathogen, was carried out in 10 cm petri dish containing 10 mL of potato dextrose agar. Fungal were incubated in 10 mL of nutrient broth in a thermal shaker for 12 h at 37 °C, and then 5 mL of this fungal suspension was transferred to 50 mL of nutrient broth and incubated for another 3–6 h in order to shift bacterial growth to the midlogarithmic phase. The fungal suspension was then centrifuged at 2000 g for 10 min, and the fungal pellet was resuspended in 1 mL normal saline and the solution was scraped on the petri dish. Sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of edge of the petri dish and to the paper was dropped aliquots of 1 mg/mL anti-fungal peptides containing PBS buffer. And the petri dishes were placed in a 37 °C incubator to allow the growth of the fungal colonies. PBS only was conducted as the negative control and amphotericin was used as the positive control.

For the more accurate activity screening based on the liquid medium fungi culture method, peptides were dissolved in 10 mM PBS (pH 7.4) to give a final concentration of 1 mg/mL, the peptide solution was scraped on the agar surface and followed by the addition of the activated fungal medium as described above. The

petri dishes were then put into the 37 °C incubator and after 24 h the numbers of fungal colonies were counted.

2.3 Results and Discussion

2.3.1 *Synthesis and Characterization of Peptide I*

Based on the crystal structure (PDB ID 2GL1), the α helix and β sheet peptides need to be joined together through two disulfide bonds to form a heterodimer. To introduce two disulfide bridges, the oxidations of thiol groups were arranged in a step by step manner to avoid nonspecific side products. As a result, the thiol groups in the corresponding Cys side chains were protected by different protecting groups so that they could be removed at the desired stages for selective oxidation (Fig. 2.2). And this strategy has found broad application in total synthesis of many difficult peptides and proteins [7].

In our strategy, trityl group was utilized to protect the first pair of Cys side chain, which could be cleaved by the TFA treatment. To increase the reactivity, as well as the specificity of the disulfide bond formation, DTNP was used to activate the thiol group in the β sheet peptide chain. The reaction was monitored by RP-HPLC (Fig. 2.3). A peak with the retention time of 17.4 min emerged and gradually increased as the reaction proceeded, accompanied by the decrease of the peak represented for pep 1. The reaction was completed in about 20 h and the intermediate peptide was purified by semi-prep HPLC. The preactivated pep 3 was mixed with the α -helix peptide in the presence of argon for a disulfide exchange reaction to give the intermediate pep 4. This reaction was completed within 1 min with good purity as monitored by HPLC. The acetaminomethyl (Acm) protecting group was removed by I_2 oxidation, with a simultaneous formation of the second disulfide bridge. However, the reaction gave complicated products as monitored by RP-HPLC. Over-oxidation might be the reason. Nevertheless, the desired product was found in the product as confirmed by LC-MS. The I_2 oxidation reaction needs further improvement. Therefore, pep 4 as well as the I_2 oxidation product was submitted to antifungal activity screening.

2.3.2 *Synthesis and Characterization of Peptide II*

It was reported that the β -loop structure containing β sheets β_2 and β_3 might harbor the antibacterial activity of defensins [8–10]. But, Cys seems to be not essential for the biological activity [11]. So we designed a small peptide library, based on the 2nd and 3rd β sheets sequence as the parental sequence (Peptide 1, sequence shown in Table 2.1, structure shown in Fig. 2.4). Given the fact that the Cys contributes little to the biological activity and with the purpose of preventing the formation of

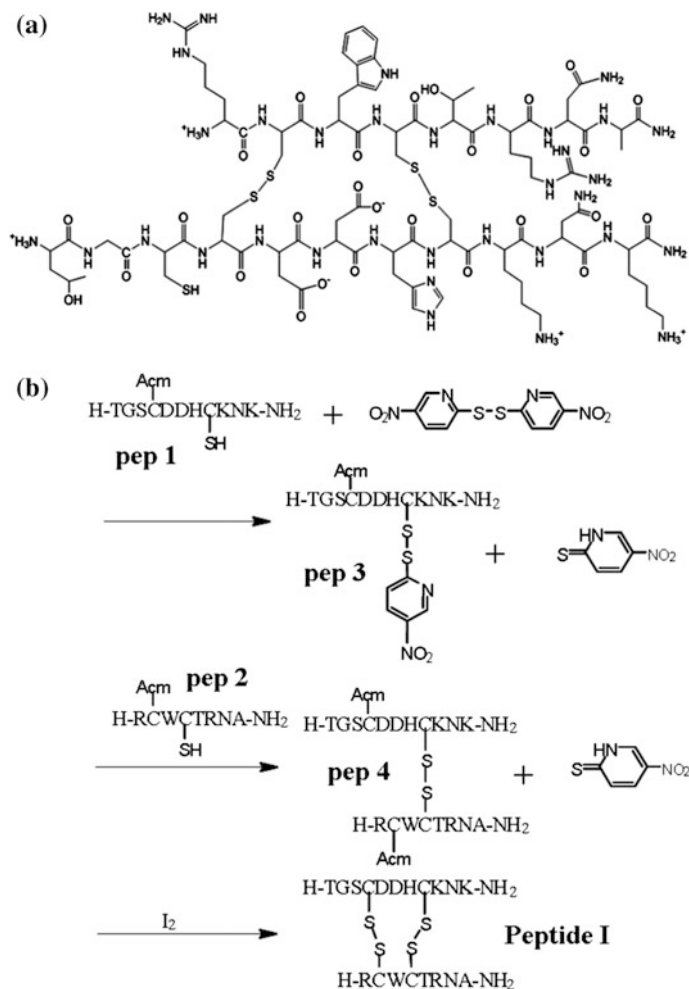


Fig. 2.2 Structural illustration of Pep I and the synthesis scheme. **a** Molecular structure of the two disulfide bonded β sheet stabilized α helix peptide. **b** Synthesis scheme of the β sheet stabilized α helix peptide

nonspecific disulfide bonded, all the three Cys were changed to Ala to yield peptide 2. As seen in the peptide sequence, two Asp were localized on the top corner of the β loop, and this structure was further stabilized by the hydrogen bonding interactions and the cation- π interactions. The two Asp were replaced by two continuous D-Pro and L-Pro, with all the Cys mutated into Ala to yield peptide 3. The sequence DPLP can force the peptide backbone to adopt a beta turn structure [8]. Furthermore, it might also reduce the negative charges of the peptide. In peptide 4, two cysteines can form an intramolecular disulfide bond to enhance the stability of the beta loop secondary structure.

Fig. 2.3 HPLC traces of the activation of Pep 1 and the conjugation of Pep 4.

a Activation reaction of pep 1 by DTNP as monitored by HPLC, the purified product was shown in green line.

b Conjugation reaction between pep 2 and pep 3 as monitored by HPLC, pep 4 was the purified product

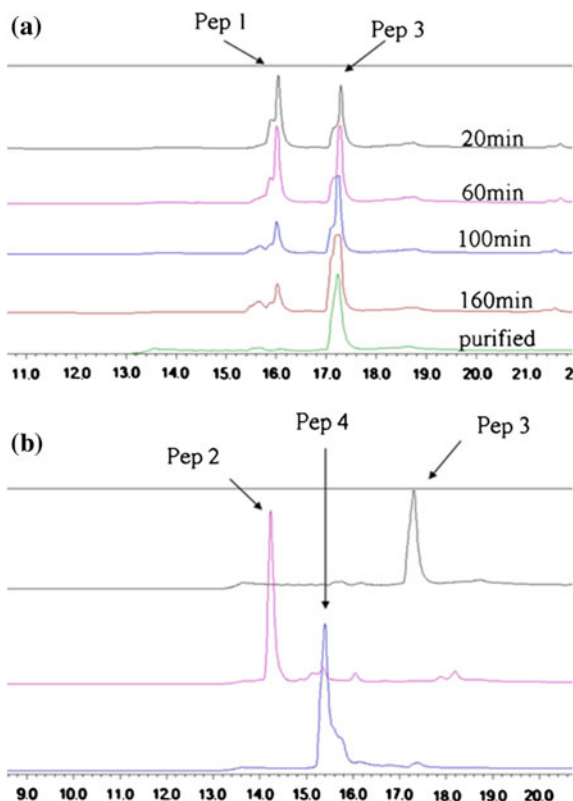


Table 2.1 Peptide sequences and mass spectral data

No.	Sequence ^a	M_{calc}	MALDI-TOF MS [M+H] ⁺	ESI MS [M+H] ⁺
1	H-KNKEHLLSGRCRDDFCWCTR-NH ₂	2622.248	2623.317	–
2	H-KNKEHLLSGRARDDFRAWATR-NH ₂	2526.332	2527.459	2528
3	H-KNKEHLLSGRAR ^D P ^L -PFRWATR-NH ₂	2490.391	2491.467	2491.6
4	H-KNKEHLLSGRCR ^D P ^L -PFCWATR-NH ₂	2552.327	2553.335	–

^aD^P represents for D-Pro, ^LP represents for L-Pro and for peptide 4, an intramolecular disulfide bond is formed

2.3.3 Structure–Activity Relationship Studies

All four peptides were prescreened for their antimicrobial activity against *Candida Albicans*. Peptides 3 and 4 inhibited the growth of the fungus, but not peptides 1 and 2. In each petri dish the fungus would grow on the paper, if the area does not contain inhibitory activity; or, if the area contains inhibitory molecules, the microbes will avoid it. As shown in Fig. 2.5, a clear blank rim could be observed in

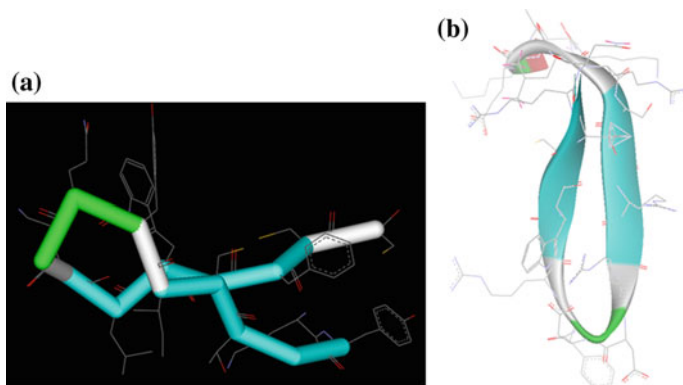


Fig. 2.4 3D structure of the β -loop peptide. Amino acids in green represent the turn structure. Basic amino acids are labeled in blue. **a** Side view. **b** Top view

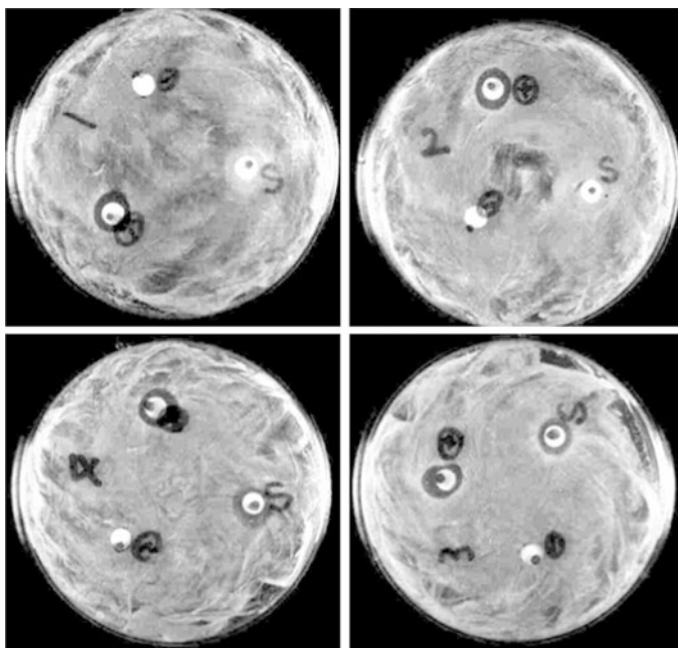


Fig. 2.5 Photographs of bioactivity screening of peptides 1–4 (Clockwise, start from top left corner). *S* stands for peptide sample. + stands for positive control. – stands for negative control

peptide 3 and peptide 4, but not in plates 1 and 2 which contain peptides 1 and 2, respectively.

To be more quantitative, peptide solutions were mixed with fungal culture and grown overnight at 37 °C in a thermal shaker. 200 μ L of the mixture was then

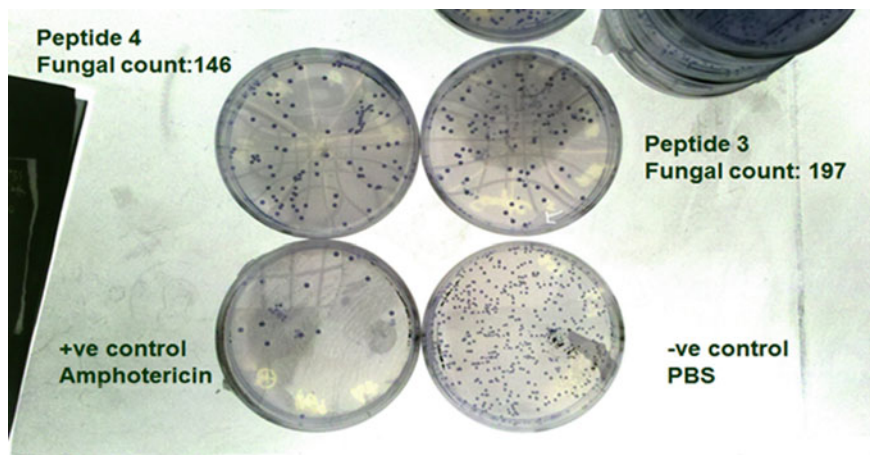


Fig. 2.6 Photographs of liquid fungal activity assay. Amphotericin and PBS buffer were used as positive and negative controls, respectively. Fungal cloning numbers were counted. (Clockwise, starting from top left corner, peptide 4, peptide 3, negative control and positive control)

spread on agar plates. After incubation at 37 °C for 24 h, colonies emerge, and were counted. Compared to the PBS negative control, plates containing peptide 3 and peptide 4 showed fewer colonies (Fig. 2.6), meaning that both peptides exhibited antifungal activities. Relatively, peptide 4 showed higher activity as evidenced by even fewer colonies (146 vs. 197).

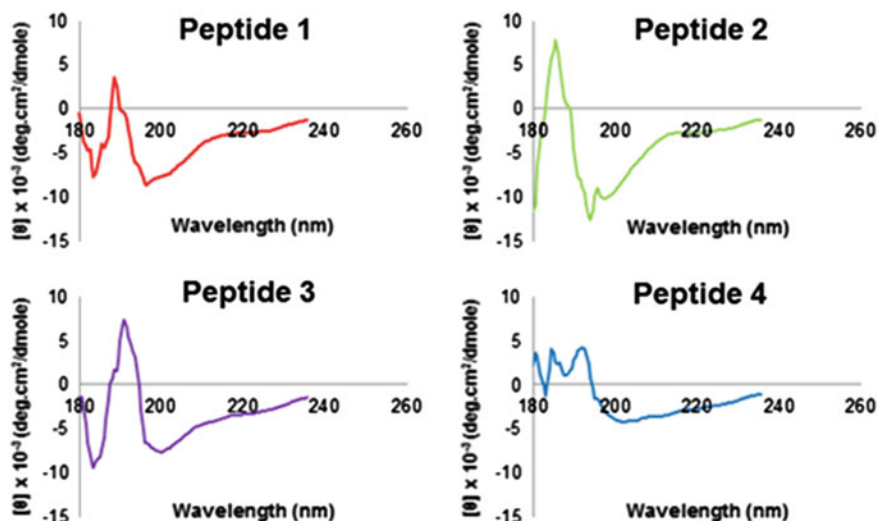


Fig. 2.7 CD spectra of the 4 peptides (Measured at the concentration of 50 $\mu\text{g/mL}$ in 10 mM PBS, pH 7.4, the curve was the average value of ten scans)

We then utilized circular dichroism spectrum to characterize the secondary structure of the four peptides to find a correlation between secondary structure and antifungal activity (Fig. 2.7). Peptide 1 and peptide 2 mainly adopted the random coil structure while peptide 3 and peptide 4 possessed the beta loop structure due to a minimum band at 205 nm.

2.4 Conclusion

Two structural motifs, a β sheet- α helix unit stabilized by two disulfide bonds and the β loop of the white cloud bean defensin have been designed based on the crystal structure. Synthesis of both structures was attempted through solid phase peptide synthesis. Iodine oxidation of AcM protected cysteines failed to yield a clean product, so still it needs further optimization. Peptides mimicking the beta loop manifested desired antifungal activity against *Candida Albicans*. This work paved the road to further optimize the beta loop sequence to search for potential antifungal activity. We only examined the fungal strain *Candida Albicans*; in future experiments, we aim to include other fungi to examine whether peptides 3 and 4 have specificity toward different strains of fungi. We will further optimize the sequence of peptides 3 and 4 by including more positively charged and aromatic amino acids to increase its membranolytic activity. The mechanism of the antifungal activity is still under investigation; most likely the peptides disrupt the membrane of the cells. One should note that the biological activity of the defensin fragments might have significantly deviated from their parental peptide. In other words, new antimicrobial activity might be discovered in further structure–function relationship studies.

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