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Functional and Structural Characterization of Apidaecin

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Drug resistance is a major obstacle to successful antibacterial chemotherapy. With the emergence of antimicrobial-resistant bacterial strains, the current drug families start to fail, and there is an urgent need for alternative agents, preferably with novel modes of action that will prevent bacteria from mounting a quick response and building resistance. Small cationic antimicrobial peptides (AMPs) are evolutionarily ancient components of the host defense system of many different unicellular and pluricellular organisms, from bacteria to plants, insects, fish, amphibians, birds, and mammals, including humans.[1] In spite of their highly diverse sequences and structural motifs, most of them show a tendency to assume amphiphilic structures in membrane environments. This feature correlates with their ability to permeable the bacterial membranes, eventually leading to lysis of the microbial cells.[2] In addition to this membrane damaging mechanism, a minority of AMPs, such as those belonging to the Pro-rich group of insects and mammals,[3] are able to kill bacteria without any apparent membrane destabilization. They can efficiently translocate inside both prokaryotic[4,5] and eukaryotic cells,[6-8] and accumulate in the cytoplasm or in other sub cellular compartments. The mechanism of cellular uptake is not yet fully understood. However, the structural similarity of these peptides to the arginine-rich cell-penetrating peptides from protein transduction domains[9] and solid experimental evidence suggested a common translocation mechanism for eukaryotic cells, *via* endocytic pathways.[8] Much less is known of the mechanism of penetration in bacterial cells, although a more specific translocation machinery has been hypothesized, involving a bacterial permease/membrane transporter.[10]

Apidaecins are the largest group of proline-rich antimicrobials known and major humoral components induced in honeybee lymph upon bacterial infection. These cationic peptides are highly stable at a low pH (2) and high temperature (100 ^{o}C). They were the first to be studied in detail with respect to the mechanism and the identity of the amino acid residues responsible for the antibacterial action.[10-14] The pharmacophore delivery unit architecture has been proposed to be a general feature of the proline-rich antibacterial peptide family.[15-16] However, despite a wealth of information on the amino acid residues important for function, little is known about the pharmacophore delivery unit architecture of apidaecin. So, in this research, full sequence of apidaecin and fragments that were gradually removed from N-terminal to C-terminal were synthesized and studied their growth inhibitory ability against microbial organisms in order to identify the functional region and secondary structure.

Experimental Section

Peptide synthesis and purification. Resin loaded with Leu- and Cys-substituted PEG-PS (0.17 mmol/g and 0.54 mmol/g respectively) were used as the support.[19] The coupling of Fmoc-amino acids was performed with an equimolar mixture of diisoproplycarbodiimide (DIPCI)/1-hydroxybenzotriazole (HOBT) as the coupling reagents. The side chain protecting group of amino acids were as follows: Asn, Cys, Gln and His, trityl; Tyr, *tert*-butyl; Arg, 2,2,5,7-pentamethylchroman-6-sulfonyl (Pmc); Glu, *tert*-butoxy. After coupling the last amino acid, the Fmoc-group was removed with 20% piperidine/DMF, and the protected side chains and peptide-resin were deprotected and cleaved with a mixture of TFA-based reagent (90% TFA, 2.5% 1,2-ethanedithiol, 2.5% triisopropylsilane, 2.5% phenol v/v) for 3 h at room temperature, and then precipitated with diethylether, and dried in the vacuum. The crude peptides were dissolved in 10% acetic acid and purified by a reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative (15 μm, 10 × 250 mm) C_{18} Bondapak column using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA).

Purity of the purified peptides was checked by a RP- HPLC on an analytical (10 μm, 4.6 × 150 mm) C_{18} Pepmap column using a water-acetonitrile gradient [30 - 70% / 30 min] containing 0.05% trifluoroacetic acid (TFA). The purified peptides were hydrolyzed with 6 N HCl at 110 ^{o}C for 24 h, and then dried in the vacuum. The residues were dissolved in 0.02 N HCl and subjected to amino acid analyzer (Hitachi Model, 835). Peptide concentration was determined by amino acid analysis.[17-18] The molecular weights of the synthetic peptides were determined by using the Micro Mass Spectrometer.

Antimicrobial activity assay. The bacterial strains were grown in Luria-Bertani medium (LB, 10 g of bactotryptone, 5 g of yeast extract and 10 g of NaCl per litre) at 37 ^{o}C overnight and diluted in a basal medium of 1% bactopeptone (Difco) to 1 : 200 [*i.e.* final bacterial suspension containing 2-4 × 10^6 colony formation units (CFU)/mL]. The peptide solutions were prepared from 200 μg/mL stock solution to give a range of 100-1.56 μg/μL in 1% bactopeptone media. Aliquots (100 μL) of a bacterial suspension at 2 × 10^6 colony-forming units (CFU)/mL in 1% pep-

tone were added to 100 μL of the peptide solution and then incubated at 37 °C overnight.

The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in optical density (OD) at 620 nm after 18 hr.

Hemolytic activity. The hemolytic activity of the peptides was determined using human erythrocytes. Fresh human erythrocytes were rinsed three times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.0), centrifuged for 15 min at 900×g, and resuspended in PBS. Aliquots (100 μL) of the suspension (0.4% in PBS, v/v) were plated in 96-well plates (Nunc, F96 microtiter plates), and then 100 μL of the peptide solution of PBS was added (the peptide final concentration: 100 μg/mL).

The plates were incubated for 1 hr at 37 °C, and then centrifuged at 1000×g for 5 min. 100 μL aliquots of the supernatant were transferred to 96-well microtiter plates (Nunc). Release of hemoglobin was monitored by measuring the absorbance at 414 nm with an ELISA plate reader. Zero percent hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively.

The hemolysis percentage was calculated by the following formula:

$$\% \text{ hemolysis} = [(Abs_{414\,nm} \text{ in the peptide solution} - Abs_{414\,nm} \text{ in PBS}) / (Abs_{414\,nm} \text{ in 0.1\% Triton-X 100} - \text{Abs in PBS})] \times 100$$

Circular dichroism (CD) studies. CD spectra of peptides were recorded using a spectropolarimeter (Jasco J715, Japan). All samples were maintained at 25 °C during analysis. Four scans per sample performed over wavelength range 190 - 250 nm at 0.1 nm intervals. The spectra were measured in 0% and 50% (vol./vol.) TFE and 30 mM SDS in Tris buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH 7.4), respectively at 25 °C using a 1 mm path length cell. The peptide concentrations were 100 μM. The mean residue ellipticity, $[\theta]$, is given in deg × cm^2 × $dmol^{-1}$: $[\theta] = [\theta]_{obs}$ (MRW/10*lc*), where $[\theta]_{obs}$ is the ellipticity measured in millidegree, MRW is the mean residue molecular weight of the peptide, *c* is the concentration of the sample in mg/mL, and *l* is the optical path length of the cell in cm. The percent helicity of the peptides was calculated with the following equation: % helicity = 100 ($[\theta]_{222} - [\theta]^0_{222}$) / $[\theta]^{100}_{222}$ where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm. Values for $[\theta]^0_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helical contents at 222 nm, are estimated to be −2,000 and −30,000 deg·cm^2/dmol, respectively.

Results and Discussion

The peptides were synthesized manually by solid phase strategy through Fmoc chemistry. In Table 1, the primary sequences of the chemically synthesized peptides used in this study are listed. The homogeneity of the synthetic peptides was confirmed by the profile of analytical HPLC on C_{18} column. The correct amino acid composition and molecular weight of the synthetic peptides were also confirmed by amino acid analysis.

The antibacterial activities of the peptides were determined by measuring minimal inhibitory concentration (MIC) of the growth against Gram-negative (*E. coli*, *S. typhimurium*, *P. aeruginosa* and *P.vulgaris*) and Gram-positive bacteria (*B. subtilis, S. pyogenes*, and *S. epidermidis*), respectively (Table 2 & 3). Each peptide was tested in triplicate in a two-fold dilution series ranging from 50 mM to 0.78 mM. Various deletion and substitution analogs were also synthesized and evaluated for their antibacterial response to ascertain the contribution of individual residues in the overall activity. The full sequence of apidaecin inhibits the bacterial growth with MIC value of only 6 μM. Removal of first three residues (Gly^1, Asn^2, Asn^3) from N-terminal of apidaecin (AP-1) did not cause much change in the antibacterial activity but removal of next three residues ($Arg,^4$ $Pro,^5$ Val^6) (AP-2) led to substantial loss of activity in *E.coli* (Table 2). The MIC is found to be increased from 0.5 μM to 32 μM in the case of gram-negative bacteria, *S.typhimrium* on deletion of $Tyr,^7$ Ile^8(AP-3), and Pro^9(AP-4) but further deletion led to complete loss of antibacterial activity in the resultant heptamer. The results obtained from the fragments PR-1, PR-2 and PR-3, synthesized based on the repeating region in apidaecin (Pro-Arg-Pro, Pro-His-Pro) showed that the PR-2 (trimer of the repeating region, PRPPHP) has similar antibacterial activity like the full sequence of apidaecin. Moreover, PR-2 and PR-3 showed more potent in activity even in other gram-negative bacteria likes *P.aeruginosa* and *P.vulgaris*.

On the other hand, the radial diffusion assay was used to evaluate the susceptibility of gram-negative and gram-positive bac-

Table 1. Amino acid sequence of apidaecin and its derivatives

Peptide	No. of amino acid	M.W	Sequence
Apidaecin	18	2107.15	GNN-RPV-YI-P-QP-RPPHPRL
AP-1	15	1822.04	RPV-YI-P-QP-RPPHPRL
AP-2	12	1470.74	YI-P-QP-RPPHPRL
AP-3	10	1194.41	P-QP-RPPHPRL
AP-4	9	1096.62	QP-RPPHPRL
AP-5	7	871.51	RPPHPRL
AP-P	28	3273.70	**RR-EAEPEAEP**-GNNRPVYIPQ-PRPPHP-RL
PR-1	14	1650.97	PRPPHP-PRPPHP-RL
PR-2	20	2332.77	PRPPHP-PRPPHP-PRPPHP-RL
PR-3	26	3014.58	PRPPHP-PRPPHP-PRPPHP-PRPPHP-RL

Table 2. Antimicrobial activities of the peptides by the microdilution broth assay

Peptide	Gram-negative bateria (MIC: μM)				Gram-positive bateria (MIC: μM)		
	E.coli	*S.typhimrium*	*P.aeruginosa*	*P.vulgaris*	*B.subtilis*	*S.pyogenes*	*S.epidermidis*
Apidaecin	6.0 <	0.5	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-1	16.0 <	0.5	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-2	64.0 <	0.5	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-3	64.0 <	16.0	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-4	64.0 <	32.0	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-5	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
AP-P	64.0 <	2.0	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
PR-1	8.0	0.5	64.0 <	64.0 <	64.0 <	64.0 <	64.0 <
PR-2	0.5	0.5	16.0 <	2.0	64.0 <	64.0 <	64.0 <
PR-3	1.0	0.5	4.0 <	2.0	32.0 <	64.0 <	64.0 <

Table 3. Comparison of antimicrobial activities of the peptides by the radial diffusion assay

Peptide	Area in mm^2 at 5 nmol (Gram-negative bateria)				Area in mm^2 at 5 nmol (Gram-negative bateria)		
	E.coli	*S.typhimrium*	*P.aeruginosa*	*P.vulgaris*	*B.subtilis*	*S.pyogenes*	*S.epidermidis*
Apidaecin	49.0	144.0	0	0	0	0	0
AP-1	20.3	81.0	0	0	0	0	0
AP-2	0	25.0	0	0	0	0	0
AP-3	0	0	0	0	0	0	0
AP-4	0	0	0	0	0	0	0
AP-5	0	0	0	0	0	0	0
AP-P	0	16.0	0	0	0	0	0
PR-1	25.0	64.0	0	0	0	0	0
PR-2	64.0	121.0	0	0	0	0	0
PR-3	72.5	121.0	0	0	0	0	0

teria to apidaecin and its derivatives. The results are summarized in Table 3. The results of an assay demonstrated that the size of the clear zone increased with the lower dose of apidaecin and AP-1 fragment, PR-2 and PR-3 (5 nmol) against *E.coli* and *S. Typhimrium*, although it varied from one strain to another. The different method by microdilution broth assay and radial diffusion assay were showing similar results against all tested strains.

The hemolysis of human erythrocytes induced by the peptides was measured. Native apidaecin and all synthetic peptides didn't exhibit any hemolytic activity against human erythrocytes up to the peptide concentration of 100 μM.

In order to investigate the relationship of structure and antibiotic activity of the peptides, the CD spectra of the peptides in phosphate buffer, and (trifluoroethanol) TFE solution or (sodium dodecyl sulfate) SDS micelles as lipid membrane-mimicking environment were measured. The CD spectra of Apidaecin and its peptide fragments revealed a random coil conformation in all tested solvent, for example the structural transformation wasn't seen even in 50% TFE or 30 mM SDS micelles. Micellar SDS has been used as a model of the negatively charged bacterial lipid membranes, with which highly cationic peptides except AP-P first interact with the hydrophobic core of the micelle. In the case of AP-P, the precursor linked to the N-terminal of apidaecin imparts a negative charge on the peptide due to the presence of higher content of glutamic acid residue. The decrease in the α-helicity of AP-P in 30 mM SDS might be due to the peptide unfolds created by electrostatic repulsion of SDS on peptide. The structural ratio of apidaecin and its derivative shown in the Table 4 was characterized by a broad negative band around 200 nm, characteristic of random coil conformation. The fragments AP-5, AP-P and PR-3 show less or no potent in anti-bacterial activity, although these fragments contain similar α-helix or β-sheet structural ratio to apidaecin and PR-2. These results suggested that the α-helicity or β-sheet of the peptide may not be the key factor in killing bacterial cells. PR-2 (trimer of repeating region of apidaecin) has more potent anti-bacterial activity than other fragments. This is likely due to the fact that the Pro backbone in the middle conserved domain is structurally required to achieve its activity. Based on our analysis we consider two structural components extremely essential for the manifestation of full spectrum of apidaecin activity, first is a polyproline frame of the backbone with consecutive PXP motives and second is the unique spatial arrangement of C-terminal conserved domain, which is responsible for stereo-specific binding with intracellular targets.

In summary, in the present study, we identified that apidaecin has effective antibiotic activities against gram-negative bacteria, but was inactive against human erythrocytes. Apidaecin and its

Table 4. Structural ratio of apidaecin and its derivatives

Peptide	Buffer			50% TFE			30 mM SDS		
	α-helix	β-sheet	β-turn	α-helix	β-sheet	β-turn	α-helix	β-sheet	β-turn
Apidaecin	0	21.7	30.0	4.5	42.6	16.2	8.1	18.7	32.9
AP-1	3.1	48.4	16.5	6.1	34.5	22.1	9.8	50.2	14.1
AP-2	0	49.2	14.9	7.3	36.8	21.7	5.7	74.9	0
AP-3	0	47.8	16.8	3.7	36.0	21.6	5.3	44.4	17.4
AP-4	3.4	23.4	32.1	5.7	42.3	18.5	20.1	49.9	0
AP-5	2.4	51.1	16.5	4.4	39.7	19.0	5.5	17.4	34.0
AP-P	25.3	7.7	46.1	4.2	32.3	20.4	17.0	22.7	24.0
PR-1	3.5	49.4	15.4	5.0	36.2	20.8	5.0	10.8	37.1
PR-2	0	31.8	26.4	4.2	39.2	19.5	11.6	6.4	42.8
PR-3	7.7	47.3	20.9	4.6	34.2	23.1	10.2	25.1	30.9

fragments such as AP-1, PR-1, PR-2 and PR-3 displayed higher antibacterial activity against *E.coli* and *S.typhimrium,* but doesn't show antimicrobial activity against gram-positive bacteria even at 64 μM. We also found that the synthetic peptide, PR-2 which contains repeating region of apidaecin displayed a potent antibacterial activity against gram-negative bacteria with no hemolytic activity at a high peptide concentration of 100 μM. In order to design the short peptides having more improved antibiotic activity than parental apidaecin, the study of the structure-activity relationships based on PR-2, that contains the functional region of apidaecin, in more detail will be necessary.

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