Anoplin, a novel antimicrobial peptide from the venom of the solitary wasp *Anoplius samariensis*

Katsuhiro Konno a,b,*, Miki Hisada c, Renato Fontana a,b, Carla C.B. Lorenzi b,d, Hideo Naoki c, Yasuhiro Itagaki c, Akiko Miwa f, Nobufumi Kawai g, Yoshihiro Nakata h, Tadashi Yasuhara i, João Ruggiero Neto b,d, Walter F. de Azevedo Jr. b,d, Mario S. Palma a,b, Terumi Nakajima c

**Abstract**

A novel antimicrobial peptide, anoplin, was purified from the venom of the solitary wasp *Anoplius samariensis*. The sequence was mostly analyzed by mass spectrometry, which was corroborated by solid-phase synthesis. Anoplin, composed of 10 amino acid residues, Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH₂, has a high homology to crabrolin and mastoparan-X, the mast cell degranulating peptides from social wasp venoms, and, therefore, can be predicted to adopt an amphipathic α-helix secondary structure. In fact, the circular dichroism (CD) spectra of anoplin in the presence of trifluoroethanol or sodium dodecyl sulfate showed a high content, up to 55%, of the α-helical conformation. A modeling study of anoplin based on its homology to mastoparan-X supported the CD results. Biological evaluation using the synthetic peptide revealed that this peptide exhibited potent activity in stimulating degranulation from rat peritoneal mast cells and broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. Therefore, this is the first antimicrobial component to be found in the solitary wasp venom and it may play a key role in preventing potential infection by microorganisms during prey consumption by their larvae. Moreover, this peptide is the smallest among the linear α-helical antimicrobial peptides hitherto found in nature, which is advantageous for chemical manipulation and medical application. © 2001 Elsevier Science B.V. All rights reserved.

Abbreviations: PMTX, pompilidotoxin; EMP-AF, eumenine mastoparan-AF; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CPY, carboxypeptidase Y; APM, aminopeptidase M; PSD, post-source decay; CID, collision-induced dissociation; CD, circular dichroism; TFE, trifluoroethanol; PC, L-α-phosphatidylcholine; PG, D-α-phosphatidyl-α-glycerol

* Corresponding author, at address a. Fax: +55-19-534-0009. E-mail address: kk-gon@rc.unesp.br (K. Konno).
1. Introduction

Solitary wasp venoms may be a rich source of bioactive substances, in particular neurotoxins, as well as spider and scorpion venoms since solitary wasps use their venoms to paralyze insects or spiders and feed the paralyzed prey to their larvae [1]. However, the chemical components of the solitary wasp venoms have been only poorly documented contrary to those of the spider and scorpion venoms. Earlier studies revealed that solitary wasp venoms indeed contain neurotoxins. Philanthotoxins are non-competitive antagonists of glutamate and nicotinic receptors found in the digger wasp venom [2,3]. Bradykinin related peptides blocking nicotinic acetylcholine receptors in insect central nervous systems have been obtained from the European scoliid wasp venoms [4,5].

We have recently surveyed the bioactive substances in the solitary wasp venoms and found novel peptide neurotoxins, pompilidotoxins (PMTXs), in the venoms of the spider wasps *Anoplius samariensis* and *Batozonellus maculifrons* [6]. PMTXs affect not only the invertebrate nervous systems, greatly facilitating synaptic transmission in the lobster neuromuscular synapse [7], but also the mammalian central nervous systems, disrupting synchronous firing in rat cortical neurons [8], which are due to the blockade of the sodium channel inactivation [9]. Structure–activity relationship studies demonstrated that the basic amino acid residues play a key role in exhibiting the potent facilitatory action in the lobster neuromuscular synapses [10]. Moreover, we have also isolated a new mast cell degranulating peptide, eumenine mastoparan-AF (EMP-AF), from the venom of the eumenine wasp *Anterhynchium flavomarginatum micado* [11]. The structure and biological activities of this peptide are similar to those of mastoparans, the mast cell degranulating peptides isolated from various social wasp venoms. Accordingly, our recent studies indicated that solitary wasp venoms may contain a variety of bioactive substances in addition to neurotoxins.

From a further investigation of the venom sac extract of *A. samariensis*, we isolated another novel peptide, designated anoplin, as one of the major components. Anoplin is composed of only 10 amino acid residues and showed both antimicrobial and mast cell degranulating activity. This is the first antimicrobial component to be found in solitary wasp venoms and the smallest among the linear α-helical antimicrobial peptides found in natural sources. We report here the isolation, structural analysis including the secondary structure and biological activities of anoplin.

2. Materials and methods

2.1. Purification

Female wasps of *A. samariensis* were collected in Sagamiko, Ibaraki, and Kyoto. The collected specimens were immediately frozen on dry ice and kept at −75°C until use. The venom sacs were dissected immediately after thawing and lyophilized.

Sixty lyophilized venom sacs were extracted (5×1 ml) with 1:1 acetonitrile–water containing 0.1% TFA (CH3CN/H2O/0.1% TFA) and the extracts were subjected to reverse-phase HPLC (Waters, Milford, MA, USA) using CAPCELL PAK C18, 10×250 mm (Shiseido, Tokyo, Japan) with linear gradient from 5% to 95% CH3CN/H2O/0.1% TFA at a flow rate of 2.5 ml/min over 30 min (Fig. 1). The peak eluted at 19.5 min was further purified by CAPCELL PAK C18, 6×150 mm with 30% CH3CN/H2O/0.1% TFA at a flow rate of 1 ml/min to give anoplin eluted at 13.5 min.

2.2. Mass spectrometry

All mass spectra were acquired on a Voyager Elite matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA) equipped with a delayed extraction source and 337 nm pulsed nitrogen laser. For the collision-induced dissociation/post-source decay (CID/PSD) spectra measurement, ions were accelerated at 20 kV and argon was introduced into the collision cell.
2.3. Amino acid sequencing

Automated Edman degradation was performed by a gas-phase sequencer PPSQ-10 (Shimadzu, Kyoto, Japan).

On-plate exopeptidase digestion was applied for peptide ladder sequencing. Carboxypeptidase Y (CPY, pH 6.0, Sequazyme kit from PerSeptive Biosystems) was used for C-terminal sequencing, and N-terminal sequencing was performed using aminopeptidase M (APM, pH 7.5, 2.5 \( \mu \)g/\( \mu l \) from Boehringer-Mannheim, Indianapolis, IN, USA).

An aqueous solution of anoplin (0.5 \( \mu l \)) and an enzyme (0.5 \( \mu l \)) were mixed on the sample plate, and after 7 min incubation at room temperature, a matrix solution (0.5 \( \mu l \) of saturated solution of alpha-cyano-4-hydroxycinnamic acid in 1:1 CH\(_3\)CN/H\(_2\)O/0.1% TFA) was added and air dried for MALDI-TOF MS analysis.

2.4. Peptide synthesis

Peptide was synthesized by stepwise solid-phase method using N-\( \beta \)-fluorenylmethoxycarbonyl (Fmoc) chemistry with TGS-RAM resin (Rapp Polymere, Tübingen, Germany) on a Shimadzu PSSM-8 peptide synthesizer (Shimadzu). All Fmoc-L-amino acids were purchased from Nova Biochem. The side chain protective groups were tert-butyloxycarbonyl for Lys, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg, and tert-butyl for Thr. Cleavage of the peptide from the resin was achieved by treatment with a mixture of TFA/phenol/thioanisole/1,2-ethanedithiol/ethylmethyldisulfide/H\(_2\)O (80:5:5:3:2:5, by volume) using 10 ml/g resin at room temperature for 8 h. After removal of the resin by filtration and washing twice with TFA, the combined filtrate was added dropwise to diethyl ether at 0\( ^\circ \)C and then centrifuged at 3000 rpm for 10 min. Thus obtained crude synthetic peptide was purified by semipreparative reverse-phase HPLC using CAPCELL PAK C\(_{18}\), 10\( \times \)250 mm with isocratic elution of 30% CH\(_3\)CN/H\(_2\)O/0.1% TFA at a flow rate of 2.5 ml/min. The homogeneity and the sequence were confirmed by MALDI-TOF MS. The HPLC elution profile of the synthetic peptide was identical with that of the natural peptide.

2.5. Vesicle preparation

L-\( \alpha \)-Phosphatidylcholine (PC) and l-\( \alpha \)-phosphatidyl-DL-glycerol (PG) were purchased from Sigma. PC and PC/PG (1:1) liposomes were prepared by the reverse-phase evaporation technique proposed by Szoka and Papahadjopoulos [12]. Phospholipids were dissolved in ether (for PC) or chloroform (for PC/PG), and dried under rotatory evaporation. The final lipid concentration was 5 mg/ml for both systems. The lipids were adhered to the flask wall and then dissolved again in mixtures of 1:1 Tris 25 mM, 150 mM NaCl/ether (for PC) or chloroform (for PC/PG) and sonicated in ice for 2 min using a titanium tip ultrasonicator. Titanium debris was removed by centrifugation (Eppendorf table centrifuge, 10 min at 14000 rpm). The fusion of the micelles was obtained by slow evaporation of the organic solvent. After incubation of this emulsion for 30 min in 45\( ^\circ \)C, unilamellar liposomes were obtained. Homogeneous liposomes were obtained by passing the solution through a polycarbonate membrane (Millipore 0.45 \( \mu m \)). The average radius of the liposomes, determined by light scattering using Dynapro equipment, was 100 nm. The phosphate concentration in the liposomes was determined by the method proposed by Barlett [13].

2.6. Circular dichroism (CD) spectroscopy

2,2,2-Trifluoroethanol (TFE) and Tris(hydroxymethyl)aminomethane (Tris) were purchased from Merck and sodium dodecyl sulfate (SDS) from Pharmacia Fine Chemicals. Water was quartz distilled and deionized. SDS was dissolved in Tris buffer 5 mM pH 8.0. CD spectra were measured over the range 190–250 nm, using a Jasco-710 spectropolarimeter (Jasco, Tokyo, Japan) coupled to a Neslab RTE111 circulating water bath. The instrument was calibrated using (+)-10-camphorsulfonic acid. Spectra were obtained at 25\( ^\circ \)C using cells with a path length of 0.5 cm. Solutes were dissolved in H\(_2\)O and CD spectra were collected at various solute concentrations. Peptide concentrations used were 62 \( \mu M \). Data points were recorded at a scan speed of 20 nm/min, bandwidth 1.0 nm, 0.5 s response and 0.1 nm resolution. Five repeat scans were accumulated to
obtain the final averaged spectra. Following baseline correction, the observed ellipticity, $\theta$ (mdeg), was converted to mean residue ellipticity $[\Theta]$ (deg cm$^2$/dmol), using the relationship $[\Theta] = 100\theta/(l c n)$ where $'l'$ is the path length in centimeters, $'c'$ is the millimolar concentration, in residues, and $'n'$ is the number of residues in the peptide. Assuming a two state model the observed mean residue ellipticity in 222 nm ($\Theta_{222}^{obs}$) was converted to percentage of $\alpha$-helix ($f_H$) using the equation:

$$f_H = \frac{\Theta_{222}^{obs} - \Theta_{222}^{c}}{\Theta_{222}^{H} - \Theta_{222}^{c}}$$

where $\Theta_{222}^{c}$ (= +640) is the complete random coil ellipticity, $\Theta_{222}^{H}$ is the mean ellipticity for complete helical conformation and is given by:

$$\Theta_{222}^{H} = -42500(1-x/n)$$

where $n$ is the chain length in residues and $x$ is the number of non H-bonded carbonyl groups in the peptide. We used $x = 3$ for carboxyamidated peptide as proposed by Rohl and Baldwin [14].

2.7. Molecular modeling

Model building was carried out using model-by-homology of the program MODELLER [15]. The atomic coordinates for mastoparan-X (PDB access code: 1A13) [16] solved by nuclear magnetic resonance (NMR) spectroscopy were used as starting model. Torsion angles of the model were taken from the original structure whenever possible. Otherwise they were taken from a standard residue library. Where necessary the model was regularized. Reasonable positions for side chains that showed considerable Van der Waals overlap were obtained in an iterative process of flipping through all $\chi$-angle rotamers. The resulting crude structure was further optimized by means of the variable target function method (VTFM) with conjugate gradient using MODELLER.

2.8. Lobster neuromuscular preparation

Neuromuscular preparations of the walking leg of lobster, *Palinurus japonicus*, were made as previously described [17]. The excitatory and the inhibitory axons innervating the stretcher muscle were isolated at the meropodite and stimulated independently. Intracellular recordings were made from the stretcher muscle by microelectrodes filled with 4 M K-acetate (10–20 MΩ). The normal solution consisted of (in mM) 468 NaCl, 10 KCl, 20 CaCl$_2$, 8 MgCl$_2$ and Tris buffer 2, adjusted to pH 7.4.

2.9. Mast cell degranulation activity

($\beta$-$\delta$-glucosaminidase assay)

Mast cells were obtained by peritoneal lavage of large (≥ 300 g) Sprague–Dawley rats. The mast cells were isolated from containing cell types by centrifugation through a cushion of Percoll as previously described [18], washed twice by resuspension and centrifugation, and finally suspended in a HEPES buffer which comprised 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 20 mM HEPES, 1 mg/ml BSA and 1 mg/ml glucose (pH 7.4).

RBL-2H3 cells were obtained from M.A. Beaven (National Institutes of Health, Bethesda, MA, USA) and grown in RPMI 1640 supplemented with 10% fetal calf serum and 100 U/ml penicillin/100 μg/ml streptomycin solution. The cells were cultured in a 96-well plate (5 × 104 cells/0.1 ml/well) in growth medium in preparation for $\beta$-$\delta$-glucosaminidase.

Degranulation was determined by measuring the release of the granule marker, $\beta$-$\delta$-glucosaminidase, which co-localizes with histamine, as previously described [18]. The cells were incubated with various concentrations of the peptide for 10 min at 37°C, and then quenched by addition of 0.5 ml of ice-cold HEPES buffer. After centrifugation, the supernatants were sampled for $\beta$-$\delta$-glucosaminidase assay. Briefly, 50 μl of samples of the medium and 50 μl of the substrate, 5 mM p-nitrophenyl-$N$-acetyl-$\beta$-$\delta$-glucosaminide in 0.2 M citrate, pH 4.5, were incubated in 96-well plates to yield the chromophore, $p$-nitrophenol. The absorbance of the colored product was assessed at 405 nm using a microtiter plate reader. The values for $\beta$-$\delta$-glucosaminidase released in the medium were expressed as the percentage of total $\beta$-$\delta$-glucosaminidase, which was determined in the cells lysed in 0.1% Triton X-100. $p$-Nitrophenyl-$N$-
acetyl-β-D-glucosaminidase was purchased from Sigma (St. Louis, MO, USA).

2.10. Hemolytic activity

Thirty microliters of red cell fractions were washed three times with physiological saline and suspended in 50 ml of physiological saline. Two hundred microliters of the prepared red cell suspension and 20 μl of a physiological saline solution, which contained the sample, were incubated for 30 min at 37°C in microplate wells. The sample absorbance was detected at 415 nm. Hemolytic activity was determined regarding 0.2% Triton X-100 activity as being 100% and physiological saline activity as being 0%.

2.11. Antimicrobial activity (determination of minimal inhibitory concentration, MIC)

The microorganisms used were: Staphylococcus aureus (ATCC 6538, ATCC 25923), Staphylococcus saprophyticus (clinical species), Bacillus subtilis (CCT 2471), Bacillus thuringiensis (wild species), Escherichia coli (CCT 1371), E. coli (ATCC 25922), Enterobacter cloacae (ATCC 23355), Proteus mirabilis (clinical species), Pseudomonas aeruginosa (ATCC 15442), and Candida albicans (UMP).

Müller–Hinton broth was from Difco. Serial dilution of peptide was prepared in sterilized water. Aliquots were placed in ELISA microplates containing Müller–Hinton broth (low-salt) or Müller–Hinton broth with 150 mM NaCl (high salt) in a final volume of 200 μl. The mixture was completed by inoculation of 10 μl of bacterial culture growing in the logarithmic phase of the microorganism as monitored by the UV absorbance at 600 nm. The final cells number (1×10^5/ml) was determined by plate counting.

The plates were incubated at 35°C and aliquots of 10 μl were removed both at the beginning of the assay and after overnight incubation, and then plated in Müller–Hinton agar. The number of colony-forming units was determined. The results were expressed as inhibition percentage of colony-forming units against a control; this control was obtained in each situation by counting the number of microorganisms introduced into the plate in the absence of peptide.

3. Results

3.1. Purification

The venom extracts of A. samariensis were subjected to reverse-phase HPLC (Fig. 1), and each fraction was tested on lobster neuromuscular synapses and its purity also examined by MALDI-TOF MS. The fraction eluted next to α-PMTX at 19.5 min showed no effect on the lobster neuromuscular synapses, but was further purified by reverse-phase HPLC under isocratic conditions to isolate anoplin. MALDI-TOF MS showed the high purity of the isolated peptide with a protonated molecular ion peak at m/z 1153.8 (MH^+, monoisotopic).

3.2. Structural analysis

Probably due to the small amounts and the high content of hydrophobic amino acids, Edman degradation gave poor results, determining only five amino acid residues from the N-terminus as Gly-Leu-Leu-Lys-Arg. Therefore, the peptide was chemically characterized by mass spectrometry. The MALDI-TOF MS analyses with the combined use of enzymatic ladder sequencing and CID/PSD spectra led to the full sequence [19]. Ladder sequencing of the purified peptide by CPY digestion showed the C-terminal se-
quence to be Ile/Leu-Ile/Leu-NH$_2$. Comparison of the CID/PSD spectra of the ladder peptide with that of the intact peptide extended the sequence to Lys/Gln-Thr-Ile/Leu-Ile/Leu-NH$_2$. Similarly, ladder sequencing by APM digestion and subsequent analyses of the CID/PSD spectra revealed the N-terminal sequence as Gly-Leu-Leu-Lys-Arg-Ile/Leu. These results suggested the entire sequence to be Gly-Leu-Leu-Lys-Arg-Ile/Leu-Lys/Gln-Thr-Ile/Leu-Ile/Leu-NH$_2$, which is consistent with the MH$^+$ peak at $m/z$ 1153.8. Acetylation distinguished Lys or Gln, indicating that position 7 was a Lys residue, and the Leu or Ile residues at positions 6, 9 and 10 were identified by the product ions due to $\beta$,$\gamma$-cleavage ($w$ ions and $d$ ions) in the CID/PSD spectra. Thus, the full sequence of anoplin was determined to be Gly-Leu-Leu-Lys-Arg-Ile-Lys-Thr-Leu-Leu-NH$_2$. The solid-phase synthesis of this peptide using Fmoc-$L$-amino acids and the HPLC comparison of the synthetic specimen with the natural peptide finally corroborated the sequence.

The chemical features of anoplin, rich in hydrophobic and basic amino acids with an amidated C-terminus, are similar to those of amphiphilic $\alpha$-helix peptides such as the mastoparans and magainins [20,21]. In fact, the amino acid sequence of anoplin has a high homology to those of mastoparan-X (MP-X) and crabrolin, the amphiphilic $\alpha$-helix peptides isolated from the venoms of Vespa xanthoptera [22] and Vespa crabro [23], respectively (Fig. 2). This class of peptides has been known to adopt an amphipathic $\alpha$-helical conformation, showing amphiphilic character under appropriate conditions, which is essential for exhibiting their biological activities [24–26]. The sequence of anoplin can be predicted to adapt an amphipathic $\alpha$-helical conformation as depicted in Fig. 3. In this view, the hydrophilic amino acid residues, Lys-4, Arg-5, Lys-7 and Thr-8, are located on one side, whereas all the hydrophobic amino acid residues, Ile and Leu, at positions 2, 3, 6, 9 and 10 are on the other side of the helix. Consequently, anoplin can be classified as an amphiphilic $\alpha$-helical peptide.

3.3. CD analysis

The secondary structure of anoplin was examined by CD spectroscopy. The CD spectra of anoplin in water or Tris buffer, and in the presence of 30% TFE and 167 $\mu$M SDS are shown in Fig. 4A. In pure water or Tris buffer, the CD spectra presented a small amount (~6%) of secondary structure. The fraction of the $\alpha$-helix increased with the increase in the TFE and SDS concentrations. The highest $f_H$ values obtained were capable of inducing 44% of the helical conformation in 30% (4.5 M) TFE while 160 $\mu$M SDS induced 55%. These values are not far from the maximum expected value (70%); however, a slight dependence of the peptide ellipticity on the concentration was observed in the presence of SDS, suggesting an $n$-merization process. A comparison of the amounts of these solutes that induce a secondary structure in the peptide shows that SDS is more efficient than TFE. The maximum helical fraction was obtained at about 160 $\mu$M of SDS versus 2 mM of TFE.

Conformation of anoplin was also examined in the presence of liposomes PC and PC/PG, the mimic
systems that are closer in structure to bacterial membranes. The CD spectra of anoplin in the presence of these liposomes are shown in Fig. 4B. In the presence of the zwitterionic model PC, the amount of the α-helix is very low, about 7%, which is similar to that in pure buffer. However, in the model system with negative charge PC/PG, the peptide presented a reasonable amount of α-helix, about 38%.

3.4. Secondary and tertiary structure of anoplin model

A molecular modeling study supported the above results of the CD analysis. Several attempts were made to crystallize anoplin using the previously described procedure to obtain crystals of EMP-AF [27,28]. However, only microcrystals were obtained and they were not suitable for X-ray diffraction analysis. Since there is no structure for anoplin, we decided to build a structural model based on homology. Anoplin has a high homology to MP-X, and the atomic coordinates for MP-X (PDB access code: 1A13) [16] solved by NMR spectroscopy were used as the starting model. The overall stereochemical quality of the final model for anoplin was assessed by the program PROCHECK [29] and indicates that 100% of the residues are in the allowed regions.

All residues fall in the helical region of the Ramachandran plot (data not shown); however, a close inspection of the hydrogen bonding pattern indicates that the residues ranging from 4 to 9 have the helix hydrogen pattern. The peptide backbone from 4 to 9 for the anoplin model adopts an amphiphilic α-helical conformation with three positively charged side chains located on one side and the hydrophobic side chains located on the other side of the amphiphilic α-helix (Fig. 5a). This model is in accordance with the CD spectra, which indicates the presence of up to 44% of helix in TFE solution and up to 55% in SDS solution. Fig. 5b shows the electrostatic potential surface for the anoplin model, indicating the concentration of the positively charged residues, Lys-4, Arg-5 and Lys-7, on one side of the molecule. The side chains of these residues extend in the same direction. The structure–activity relationship studies of the mastoparans have shown that positive charges are crucial for the regulatory activity on the G-proteins [16,30]. Three lysine residues are conserved in the mastoparans isolated from social wasp venoms. Two of these are kept in anoplin and a modification from lysine to arginine is observed, nevertheless, keeping a positive residue in this position.

3.5. Biological activities

The biological activities of anoplin were investigated using the synthetic peptide. The mast cell degranulation, hemolytic and antimicrobial activities were tested because they are characteristic biological activities for amphiphilic α-helical peptides.

Fig. 6 shows the results of the mast cell degranulation activity. As expected, anoplin stimulated the degranulation from the rat peritoneal mast cells. The potency was about two thirds that of mastoparan, which seems to be comparable to crabrolin because the potency of crabrolin is reported to be somewhat
less than that of mastoparan [23]. On the other hand, anoplin showed no degranulation activity on RBL-2H3 cells (data not shown). The hemolytic activity of anoplin in human erythrocytes was quite low or virtually inactive. The potency was less than 20% that of mastoparan. Crabrolin is reported to be four times less active than mastoparan in guinea pig erythrocytes [23] and showed no effect in rat erythrocytes [32]. Thus, anoplin and crabrolin seem to have similar activity profiles for mast cell degranulation and hemolysis.

The antimicrobial activity was examined both in low- and high-salt media and summarized in Table 1. In low-salt media, anoplin showed broad-spectrum inhibitory activity against both Gram-positive and Gram-negative bacteria, but was inactive to *B. thuringiensis*, *E. cloacae* (ATCC 23355) and *P. mirabilis*. The MIC values were in the range of 5–50 μg/ml which is somewhat selective to Gram-positive bacteria. This profile is in contrast to that of crabrolin, which is more selective to Gram-negative bacteria than to Gram-positive bacteria [32]. The antimicrobial activity of anoplin was salt-sensitive as has been known for defensins [33]. In high-salt media (150 mM NaCl), the Gram-negative bacteria were virtually resistant to anoplin and the potency to the Gram-positive bacteria was significantly reduced.

Table 1
Antimicrobial activity of anoplin

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (μg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>low-salt</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 25923</td>
<td>5</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 6538</td>
<td>50</td>
</tr>
<tr>
<td><em>S. saprophyticus</em></td>
<td>5</td>
</tr>
<tr>
<td><em>G(+) rods</em></td>
<td></td>
</tr>
<tr>
<td><em>B. subtilis</em> CCT 2471</td>
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</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>G(–) rods</em></td>
<td></td>
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<tr>
<td><em>E. coli</em> CCT 1371</td>
<td>50</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 25922</td>
<td>50</td>
</tr>
<tr>
<td><em>E. cloacae</em> ATCC 23355</td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>&gt;200</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 15442</td>
<td>20</td>
</tr>
</tbody>
</table>

*Low-salt medium: Müller–Hinton medium.*

*High-salt medium: Müller–Hinton medium with 150 mM NaCl.*
compared with those in low-salt media. These results indicated that the main targets of anoplin are the cytoplasmic membranes similar to defensins [34].

4. Discussion

Anoplin is one of the major peptide components of the venom of *A. samariensis*, from which we already isolated the novel neurotoxic peptide *α*-PMTX [8]. The sequence of anoplin was determined by mass spectrometry, which turned out to be classified into amphiphilic *α*-helical peptides. This class of peptides is widely distributed in venomous animals, for example, in hornet venoms [20] and in frog skin [21]. In the solitary wasp venom, we have recently found such a peptide, EMP-AF, from *A. flavomarginatum micado* for the first time [13]. Accordingly, solitary wasp venoms may be a rich source of this type of peptides.

A characteristic chemical feature of this class of peptides is that they can adapt an amphipathic *α*-helical conformation, showing amphiphilic character, which is essential for exhibiting their biological activities [24–26]. The sequence of anoplin can be predicted to adapt an amphipathic *α*-helical conformation (Fig. 3), and in fact, the CD analysis indicated the presence of up to 44% of *α*-helix in TFE solution and 55% in SDS solution (Fig. 4A). A molecular modeling study supported these results. For the anoplin model, the peptide backbone from residues 4–9, which corresponds to 60% of the anoplin molecule, adopts an amphiphilic *α*-helical conformation (Fig. 5a). This model presents a fraction of the *α*-helix close to the observed values obtained from the CD analysis. The electrostatic potential surface for the anoplin model indicated the concentration of the positively charged residues, Lys-4, Arg-5 and Lys-7, on one side of the molecule (Fig. 5b). The CD spectra also demonstrated that anoplin switches from unordered forms to a stable *α*-helical conformation when introducing TFE or SDS into the solution, indicating that TFE and SDS stabilize the *α*-helical conformation. Especially interesting is a slight dependence of the peptide ellipticity on the concentration in the presence of SDS. This observation suggests an *n*-merization process for anoplin, in accordance with previous studies, which indicate that MP-X forms a tetrameric aggregate in the presence of DMPC vesicles [35]. The biological relevance of the aggregation observed for MP-X and anoplin need further structural and functional studies.

The amphiphilic *α*-helical peptides exhibit mast cell degranulating, hemolytic and antimicrobial activities. Anoplin showed potent degranulating activity from rat peritoneal mast cells and broad-spectrum antimicrobial activity. Therefore, this peptide is the first antimicrobial component to be isolated from the solitary wasp venom, which indicates that solitary wasp venoms may be a new source of antimicrobial substances. The recent study revealed that a spider venom also contains antimicrobial *α*-helical peptides, lycotoxins, and suggested that they may play a dual role for prey capture and to prevent potential infection by microorganisms arising from prey ingestion [36]. For the solitary wasp *A. samariensis*, these two roles may be divided into two distinct peptide components; that is, *α*-PMTX is responsible for prey capture and anoplin prevents potential microbial infection during prey consumption by their larvae.

Antimicrobial peptides are widely distributed in plants, insects, amphibians and mammals, playing an important role in host defense mechanisms [37–39]. They have attracted much attention as a novel class of antibiotics, especially for antibiotic-resistant pathogens, because they non-selectively interact with...
cell surface membranes [40]. The results from the CD analysis in the presence of liposomes and the antibacterial activity in high-salt media indicated that anoplin also interacts with cell surface membranes of bacteria. Further studies, however, are needed to define the mechanism of action in more detail.

Anoplin is a rather small peptide compared to the known antimicrobial peptides; it is composed of only 10 amino acid residues, while most others have 15–40 residues. To our knowledge, anoplin is the smallest antimicrobial peptide among those hitherto found in natural sources. Additionally, it has no disulfide bonds which are involved in the majority of such peptides [41]; in other words, anoplin has a very simple chemical structure. This is advantageous for chemical modification and structure–activity relationship studies as well as for investigating its mode of action, which may be useful for the development of a novel class of antibiotics.

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