Amino Acids (2011) 40:61–68
DOI 10.1007/s00726-009-0449-y

ORIGINAL ARTICLE

Amino acid substitutions in an alpha-helical antimicrobial arachnid peptide affect its chemical properties and biological activity towards pathogenic bacteria but improves its therapeutic index

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Received: 26 October 2009 / Accepted: 10 December 2009 / Published online: 24 December 2009

Abstract Four variants of the highly hemolytic antimicrobial peptide Pin2 were chemically synthesized with the aim to investigate the role of the proline residue in this peptide, by replacing it with the motif glycine-valine-glycine [GVG], which was found to confer low hemolytic activity in a spider antimicrobial peptide. The proline residue in position 14 of Pin2 was substituted by [V], [GV], [VG] and [GVG]. Only the peptide variant with the proline substituted for [GVG] was less hemolytic compared to that of all other variants. The peptide variant [GVG] kept its antimicrobial activity in Muller–Hilton agar diffusion assays, whereas the other three variants were less effective. However, all Pin2 antimicrobial peptide variants, were active when challenged against a Gram-positive bacteria in Muller–Hilton broth assays suggesting that chemical properties of the antimicrobial peptides such as hydrophobicity is an important indication for antimicrobial activity in semi-solid environments.

Keywords Antimicrobial peptide · Arachnid, pandinin 2 · Peptide synthesis · *S. aureus*

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Abbreviations

CD	Circular dichroism
OD	Optical density
Oxki2	Oxypinin 2
Pin2	Pandinin 2
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
MIC	Minimum inhibitory concentration

Introduction

Amphipathic and cationic α-helical peptides are broadly found in animals as part of their biological defense system (Andreu and Rivas 1998). A large number of amphiphilic and cationic peptides have been characterized in invertebrates, especially from the phyla Arthropoda, and in vertebrates from the class Amphibia. Short linear cationic peptides with α-helical conformation share some common characteristics such as antimicrobial activities at low micromolar concentrations and α-helix formation in hydrophobic environments (Andreu and Rivas 1998).

Pandinin 2 (Pin2) is a 24 mer α-helical antimicrobial peptide from the venom of the African scorpion *Pandinus imperator*. This peptide has antimicrobial activities towards Gram-positive and Gram-negative bacteria in the micromolar range; however, it shows hemolytic activity at similar concentrations (Corzo et al. 2001). High-resolution structure of Pin2 determined by NMR showed that the peptide is essentially α-helical with a kink in the middle of the structure. The proline kink is a structural characteristic of some pore-forming peptides that confer a high pore-forming ability to antimicrobial peptides. Clear examples

Table 1 Amino acid sequences of Pin2, Pin2 variants, Oxki2 and ponericin G1

Peptide	Amino acid sequence	Mass (Da)	Hydrophobicity (GRAVY)[a]	Elution time (min)[b]
Pin2	FWGALAKGALKLI**P**SLFSSFSKKD	2,612.1	0.329	38
Pin2 [P14V]	FWGALAKGALKLI**V**SLFSSFSKKD	2,614.1	0.571	49
Pin2 [P14GV]	FWGALAKGALKLI**GV**SLFSSFSKKD	2,671.1	0.532	41
Pin2 [P14VG]	FWGALAKGALKLI**VG**SLFSSFSKKD	2,671.1	0.532	46
Pin2 [P14GVG]	FWGALAKGALKLI**GVG**SLFSSFSKKD	2,728.2	0.496	37
Ponericin G1	GWKDWAKKAGGWLKKK**GPG**MAKAALKAAMQ	3,212.9	−0.683	ND
Oxki2b	GKFSGFAKILKSIAKFFK**GVG**KVRKQFKEASDLDKNQ	4,146.8	−0.546	ND

ND Not determined

[a] The GRAVY (Grand Average of Hydropathy) value for a peptide or protein is calculated as the sum of hydropathy values (Kyte and Doolittle 1982) of all the amino acids, divided by the number of residues in the sequence

[b] Elution times under the HPLC conditions of Fig. 1

of such peptides are mellitin, alamethicin and pardaxin. In these peptides the presence of a proline was correlated to a high antimicrobial activity; nevertheless, these antimicrobial peptides also show high hemolytic activities (Dempsey et al. 1991; Thennarasu and Nagaraj 1996; Oren and Shai 1997; Dathe et al. 1998). Structural substitutions of proline in the amino acid sequence of these peptides had different effects. For example, substitutions of the P14 for alanine in the antimicrobial peptides mellitin and pardaxin increase their hemolytic and antimicrobial activities, respectively, as result of an increment in the helicity of their analogs (Dempsey et al. 1991). On the other hand, cationic antimicrobial peptides such as oxypinins from the wolf spider (*Oxyopes takiobus*) and ponericins G1 from the ponerin ant (*Pachycondyla goeldii*) showed antimicrobial activities with low cytotoxic effects towards erythrocytes (Orivel et al. 2001; Corzo et al. 2002). These peptides have a different amino acid motif in the middle of their primary structures. For example, oxypinin 2 has a GVG motif and ponericin G has glycine residues flanking the central proline, that is, has the GPG motif (Table 1). Moreover, cecropin and melittin synthetic hybrids in which the proline of melittin was changed by glycine or was flanked by the same residues showed no hemolytic activities related to the parental peptides and others in which the proline remains present (Shin et al. 1999). Therefore, it was hypothesized that the proline residue (P14) at the center of the structure of Pin2 is responsible for such activity. Based on low hemolytic activity shown by two α-helical peptides from the venom of the spider *O. takiobus* and *P. goeldii*, synthetic variants of Pin2 were constructed with the aim of decreasing its hemolytic activity. The antimicrobial spider peptide Oxki2 has a GVG motif and the antimicrobial ponericin has a GPG motif in the middle of their structure. These natural analogs prompt us to design a low hemolytic peptide with a GVG motif. Therefore, four variants P14V, P14VG, P14GV and P14GVG were chemically synthesized and assayed. In this work, we report the chemical and biological characteristics of such Pin2 variants.

Materials and methods

Microorganisms

Staphylococcus aureus ATCC 25923, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 6399 were purchased directly from the American Type Culture Collection through The Global Bioresource Center™ by UNAM. The strains *Streptococcus agalactiae* and *Shigella flexneri* were a donation from a local clinic and identified in our laboratory.

Peptide synthesis of Pin2 variants

Pin2 and its variants were chemically synthesized by a solid-phase method using the Fmoc methodology on an Applied Biosystems 433A peptide synthesizer. Fmoc-Gln(tBu)-PEG resin (Watanabe Ltd., Hiroshima, Japan) was used to provide a free carboxyl group at the C-terminus of Pin2 and its variants. Cleavage and deprotection of peptide resins were performed using a chemical mixture composed of 1 g crystalline phenol, 0.2 g imidazole, 1 ml thioanisol, 0.5 ml 1,2-ethanedithiol in 20 ml trifluoroacetic acid (TFA). The resin was removed by filtration, and the deprotected peptides in solution were precipitated using cold ethyl ether. The precipitated peptide was washed twice with cold diethyl ether to remove remaining scavengers and protecting groups. The crude synthetic peptide was then dissolved in a 25% aqueous acetonitrile solution and separated by reverse-phase HPLC on a semipreparative C_{18} column (10 × 250 mm, Nacalai Tesque, Japan). Cation exchange chromatography and C_{18} analytical

reverse-phase HPLC were further used to purify the synthetic peptides. The structural identities of the synthetic peptides were verified by automatic Edman degradation and mass spectrometry using a Finnigan LCQ^{DUO} electrospray ionization ion trap mass spectrometer (San Jose, CA, USA).

Circular dichroism (CD) measurements

Circular dichroism spectra were obtained on a Jasco J-725 spectropolarimeter (Jasco, Japan). The spectra were measured from 260 to 190 nm in 60% trifluoroethanol (TFE), pH 7.1 at room temperature, with a 1 mm path-length cell. Data were collected at 0.1 nm with a scan rate of 100 nm/min and a time constant of 0.5 s. The concentration of the peptides was 40 μg/ml. Data were the average of three separate recordings. Percentages of α-helix content were calculated from the spectra using the software CDNN, v. 2.1 (Bohm et al. 1992).

Antimicrobial and hemolytic assays

Minimal inhibitory concentrations (MIC) and growth inhibition curves were obtained using pure peptides. Briefly, the inoculums were prepared from fresh bacteria cultures. The minimum inhibitory concentration (MIC) values were defined as the lowest concentration of peptide at which 100% growth inhibition was observed. The cell count for each bacteria tested was 1.2×10^8 CFU/ml in Mueller–Hilton agar according to the Clinical and Laboratory Standards Institute (CLSI, http://www.clsi.org) recommendations. Serial dilutions of peptides were arranged at concentrations of 9.4, 18.8, 37.5, 75, 150 and 300 μM.

For growth curves, the final volume in each vial was 200 μl and the cell count was 1×10^5 CFU/ml for *S. aureus* at the initial time. Serial dilutions of peptides were arranged, and an aliquot of cell suspension was added to each vial. The final peptide concentration ranged from 0.39, 0.78, 1.56, 3.13, 6.25 and 12.5 μM. The optical density (OD) of each vial was measured at 625 nm in an ELISA reader (BioRad, model 450, Hercules, CA, USA). The positive control contained only the bacterial suspension, and the negative control contained only sterile culture medium.

Hemolytic activity was determined by incubating suspensions of human red blood cells with serial dilutions of each selected peptides. Red blood cells were rinsed several times in PBS by centrifugation for 3 min at 3,000*g* until the OD of the supernatant reached the OD of the control (PBS only). Red blood cells were counted by an hematocytometer and adjusted ca to $7.7 \times 10^6 \pm 0.3 \times 10^6$ cells/ml. Red blood cells were then incubated at room temperature for 1 h in 10% Triton X-100 (positive control), in PBS (blank), or with amphipathic peptides at concentrations of 0.39, 0.78, 1.6, 3.1, 6.2, 12.5 and 25 μM. The samples were then centrifuged at 10,000*g* for 5 min, the supernatant was separated from the pellet, and its absorbance measured at 570 nm. The relative optical density compared to that of the suspension treated with 10% Triton X-100 defined the percentage of hemolysis.

Statistical analysis

The last significant difference method was used to determine whether statistically significant differences occurred among the mean values obtained. The hemolysis constants (IC_{50}) were obtained using a non-linear regression where the data was fit the Boltzmann sigmoidal equation using the software package Prism 4 (GraphPad, Inc.).

Results

Peptide synthesis of Pin2 variants

Chemical synthesis of Pin2 and Pin2 variants was performed in 0.1 mmol Fmoc chemistry. The theoretical yield for 0.1 mmol peptidyl resin was 260 mg of crude synthetic peptide. Pin2 and Pin2 variants were purified by reverse-phase HPLC (rpHPLC) as describe in "Material and methods". The final yields after chromatographic purification of Pin2 variants were 40, 38, 36, 36 and 34% for Pin2, P14 [V], P14 [VG], P14 [GV] and P14 [GVG], respectively. Since a proline residue was substituted from the original amino acid sequence, the chromatographic elution times were different (Fig. 1). All Pin2 variants eluted at close retention times, and before 60% acetonitrile (solvent B) was reached under the HPLC conditions already described. All purified Pin2 variants were proved to have the expected molecular masses. For instance, the experimental versus the theoretical masses expected were 2,612.1/2,612.0 for the native Pin2, 2,614.1/2,614.1 for P14 [V], 2,671.1/2,671.0 for P14 [VG], 2,671.1/2,671.0 for P14 [GV], and 2,728.2/2,729.0 for P14 [GVG], respectively. With all five chemically synthesized peptides structural analyses as well as antimicrobial and hemolytic experiments were performed.

Secondary structure

The CD spectra of Pin2 variants were compared. The secondary structure analysis was performed using CD spectral data from 190 to 260 nm. Pandinin 2 and Pandinin 2 variants showed an unordered structure in aqueous solution (data not shown). However, in the presence of 60% TFE, the CD spectra of all peptides changed

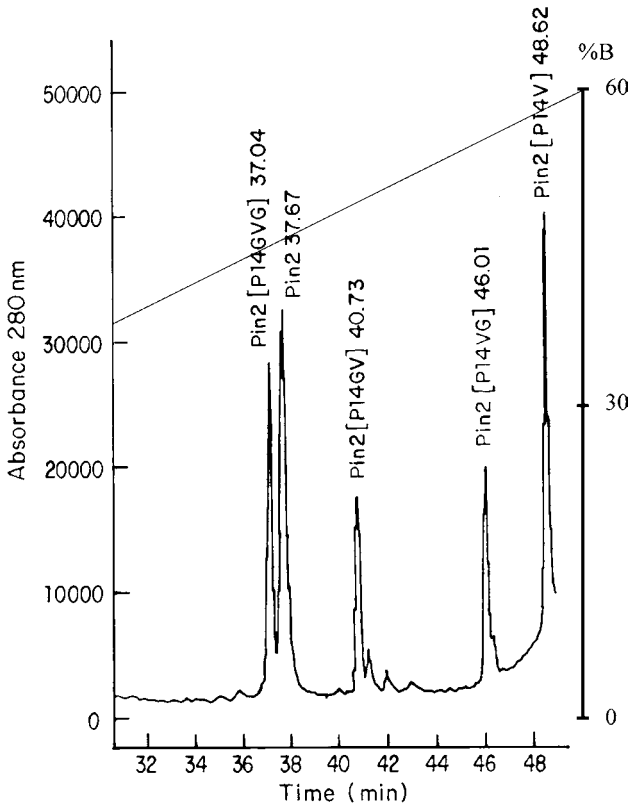

Fig. 1 Elution times of Pin2 and its variants. The peptides (10 nmol each) were fractionated using a reverse-phase analytical C_{18} column ($5C_{18}$MS, 4.6 × 250 mm) equilibrated in 15% acetonitrile containing 0.1% TFA for 5 min, and eluted with a linear gradient of acetonitrile from 15 to 60% in 0.1% TFA, run for 45 min (1%B/min) at a flow rate of 1 ml/min

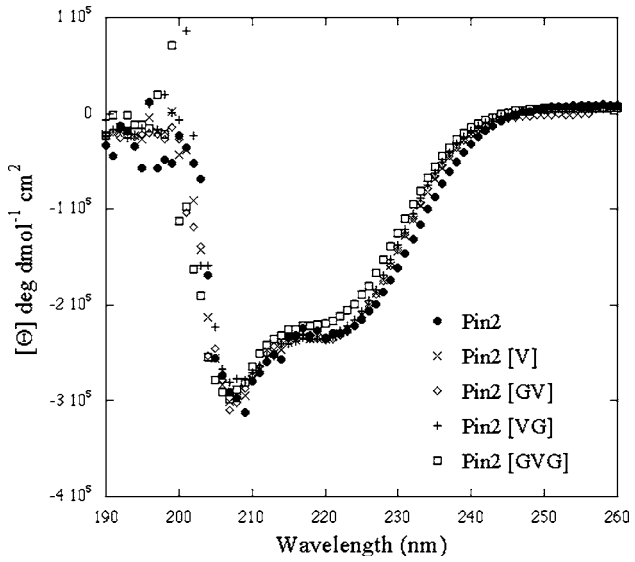

Fig. 2 Circular dichroism spectra of Pin2 and its variants in 60% TFE. The concentration of the peptides was 670 μg/ml. The alpha-helical values of the parental peptide Pin2 and the Pin2 variants were more than 98%. Data is the average of three separate recordings

dramatically and indicated a more ordered structure with a minimum ellipticity at 208 and 222 nm (Fig. 2). These data are consistent with formation of a α-helix in aqueous TFE or in a membrane-mimetic environment, as observed previously with other amphipathic antimicrobial peptides (Mor et al. 1991; Wong et al. 1997; Batista et al. 1999; Amiche et al. 2000).

Antimicrobial activity

Growth in solid culture medium

In solid culture media (agar), the antimicrobial activity of Pin2 and Pin2 variants were tested against the Gram-positive bacteria *Bacillus subtilis, Streptococcus agalactiae, Staphylococcus aureus* and *Listeria monocytogenes.* They were also tested against the Gram-negative bacteria *Shigella flexneri, Escherichia coli* and *Salmonella typhi*. Table 2 shows the minimum inhibitory concentration according to the Clinical and Laboratory Standards Institute (CLSI). Under our bioassay conditions, Pin2 displayed strong activity, with few variations between the target microorganisms. Pandinin 2 variants had weak activity against all bacteria tested except the variant Pin2 [GVG], which showed clear activity against all bacteria tested. Indeed, Pandinin 2 was almost twice more active against *S. aureus*, *S. agalactiae*, *L. monocytogenes* and *E. coli* than Pin2 [GVG].

Growth in liquid culture medium

In liquid culture media, the antimicrobial activity of Pin2 and Pin2 variants were tested only against *S. aureus*. This Gram-positive bacterium is one of the main concerns in several topical infections. The growth of *S. aureus* was observed under serial dilutions of all antimicrobial peptides from 0.4 to 12.5 μM. Figure 3a shows only the growth curves of *S. aureus* under 12.5 μM for each Pin2 variants. Under our bioassay conditions, Pin2 and all Pin2 variants displayed strong activity against this pathogenic bacterium, with few variations. An unexpected result was the variant Pin2 [V], which did not have antimicrobial properties at concentrations higher than 300 μM in solid agar media, but it has comparable activity to all Pin2 variants at 12.5 μM. These results show that amino acid modification at the proline position is irrelevant for the antibiotic activity towards *S. aureus*.

Hemolytic assays

Assays of Pin2 and Pin2 variants on human erythrocytes showed that Pin2 had the highest hemolytic activity of all of them, with 91% hemolysis observed at 25 μM for Pin2 but only 52% for Pin2 [GVG] at a similar concentration

Table 2 Minimum inhibitory concentrations of Pin2 and Pin2 variants

Strain	Gram (±)	Minimum inhibitory concentrations (μM)				
		Pin2	P14V	P14GV	P14VG	P14GVG
S. aureus ATCC 25923	+	37.5	NA	300	300	75.0
B. subtillis ATCC 6633	+	37.5	NA	300	300	37.5
S. agalactiae	+	18.8	NA	300	300	37.5
L. monocytogenes ATCC 7644	+	18.8	NA	300	300	37.5
E. coli ATCC 25922	-	18.8	NA	300	300	37.5
S. flexeri	-	37.5	NA	300	300	37.5
S. typhi ATCC 6399	-	37.5	NA	300	300	37.5

NA No antimicrobial activity

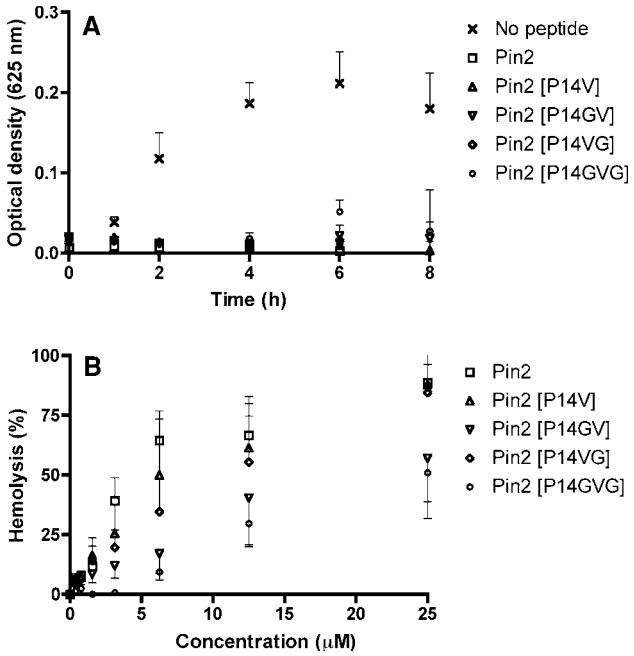

Fig. 3 Biological activity of Pin2 and Pin2 variants. **a** Antimicrobial activity was assayed using *S. aureus*. The concentration of peptides used was 12.5 μM**. b** Hemolytic activity was measured using human red blood cells. Data are the average of at least four independent experiments. *Error bars* represent the standard deviations

(Fig. 3b). Similarly, the variant Pin2 [GV] had lower hemolytic activity compared to the more hydrophobic variants Pin2 [V] and [VG]. Moreover, it can be observed that the difference in hemolysis between the parental peptide Pin2 and its variants is conserved from 0.78 to 25 μM; that is, the cell membrane disruption of such peptides seems to be unaffected by the peptide concentration.

Helical wheel projections

To understand schematically the amino acid substitutions in Pin2, helical projections of Pin2 and Pin2 variants were generated (Fig. 4). First, both helical wheels of Pin2 and Pin2 [V] look similar with the exception that the hydrophobic residue Val replaces the hydrophilic residue Pro. This replacement introduces a hydrophobic moiety in the hydrophilic face of Pin2 (left side of the helical wheel); therefore, it could be observed that such substitution increases the hydrophobic section in Pin2 [V], which is correlated with its low diffusion in agar media. On the other hand, looking at helical wheel of the Pin2 variant [GVG], it could be observed that the hydrophilic part of this peptide (left side) has been crowded with hydrophobic residues. However, it is also observed that the hydrophobic face (right side of the helical wheel) has been disrupted because of the inclusion of hydrophilic residues such Gly15 and Ser17. Moreover, other residues such Ser23 and Ser20 in such hydrophobic face may also balance the amphipathic properties of Pin2 [GVG]. Such equilibrium between the left and right side of the helical wheel seems to improve the antimicrobial activity of Pin2 [GVG] as observed in Table 2. In a quantitative basis the grand average of hydropathy (GRAVY) values of the hydrophobic face of Pin2 (FLLLAWIAFLSKFD) and Pin2 [GVG] (FLLGAWIASL-FSSK) were 1.61 and 1.25, respectively (Fig. 4). Therefore, Pin2 [GVG] is less hydrophobic than the parental Pin2 as observed in Fig. 1. Finally, the peptide variants [VG] and [GV] as well as the variant [GVG] also introduce hydrophilic residues in the right side of the helical wheel with the exemption that the variant [VG] has a hydrophobic neighbor Phe21 besides the par Ile13Val14, which make it a little more hydrophobic than that of [GV].

Discussion

Proline residues can be found frequently at the midpoint regions of α-helical components of trans-membrane proteins, where it shows a significant concentration in the central part of the amino acid sequence of trans-membrane α-helices, and it is often found prior to glycine residues

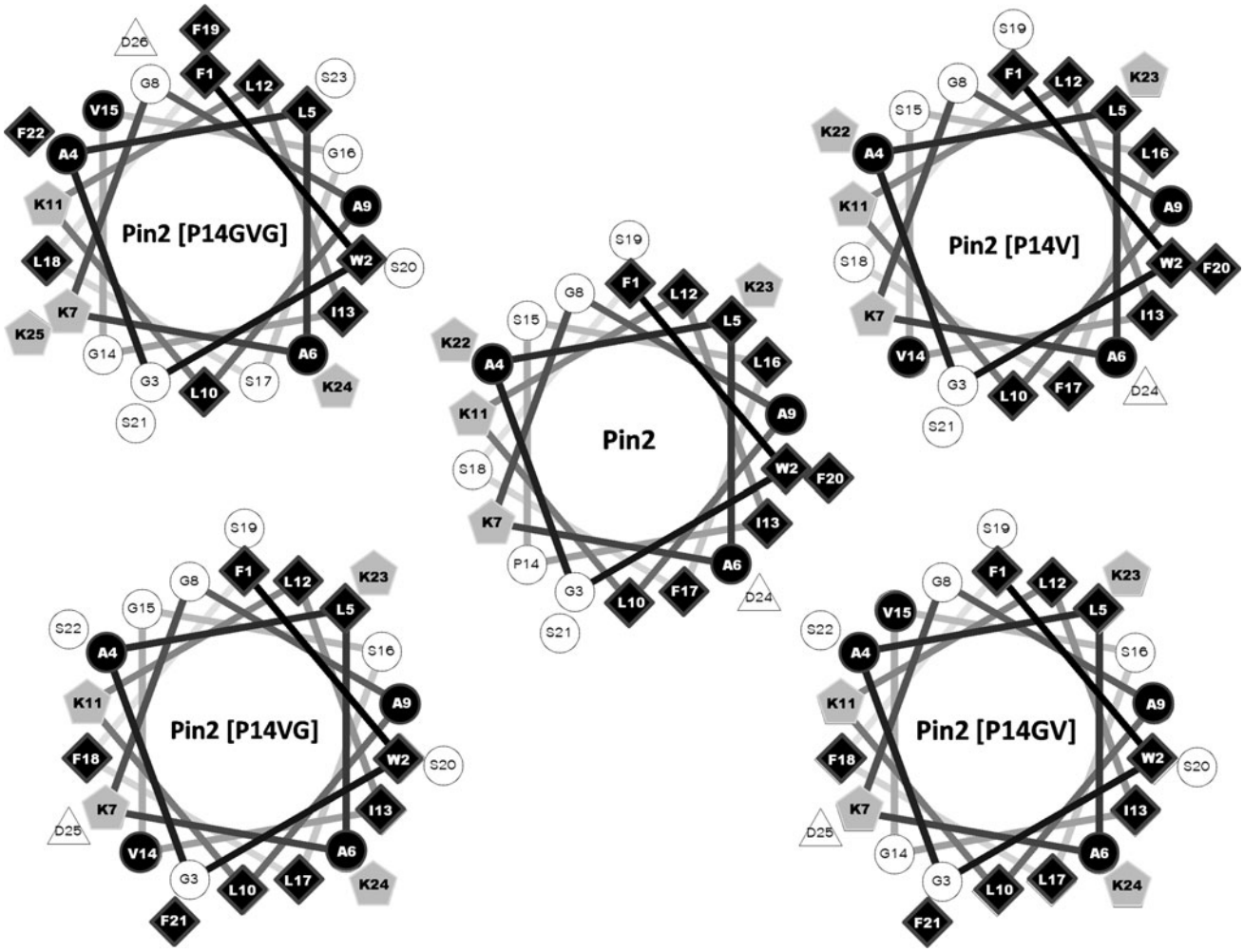

Fig. 4 Helical wheel diagrams of Pin2 and Pin2 variants. Helical wheels were prepared by the software Helical Wheel Projections; wheel.pl, v 1.3 (Zidovetzki et al. 2003). The hydrophobic, hydrophilic and neutral residues are colored in *black, gray and white*, respectively

(Sansom 1992; Bywater et al. 2001). Due to steric hindrance and the loss of hydrogen bonds, the proline residue introduces a region of flexibility in the trans-membrane structure, which is sometimes increased by the presence of glycine residues; therefore, the high frequency of proline residues in this kind of proteins implicates important structural and functional roles (Cordes et al. 2002). Similar to trans-membrane α-helices, proline plays an important role in the biological activity of the amphipathic and cationic α-helical peptides. Both the chemical synthesis and the purification of Pin2 and Pin2 variants showed different chemical properties of proline substitution by the residues [V], [GV], [VG] and [GVG] in Pin2. According to the elution times under reverse-phase HPLC, the proline substitution by [V] produces a more hydrophobic peptide. This result was expected because valine is a more hydrophobic residue than proline; however, the addition of a glycine residue to form Pin2 [VG] and Pin2 [GV] produces a less hydrophobic antimicrobial Pin2. Although the theoretical GRAVY Index of Pin2 [VG] and Pin2 [GV] are the same, experimentally the hydrophobicity of Pin2 [VG] is higher than that of Pin2 [GV] because the existence of a hydrophobic patch formed by residues I13V14 is formed in Pin2 [VG] (Fig. 4). Indeed, only the parental Pin2 and the variant [GVG] have hydrophilic clusters constituted by G3P14S21 and G3G14S21 in Pin2 and Pin2 [GVG], respectively (Fig. 4). The proline substitution by [GVG] re-established the ratio between the hydrophilicity and hydrophobicity in Pin2 [GVG]. The hydrophobicity of Pin2 and Pin2 variants are correlated to their biological activities in the agar diffusion assays because the more hydrophobic variants [V], [GV] and [VG] had lower antimicrobial properties than that of the less hydrophobic Pin2 and Pin2 [GVG]. In other words, Pin2 [V], [VG] and [GV] were less active in solid culture assays, and only the variant Pin2 [GVG] had important biological activity. Although all four variants conserved their secondary structure, the peptide Pin2 [V] showed no antimicrobial activity against any

Table 3 Therapeutic index of Pin2 and Pin2 variants for *S. aureus*

Peptide	Hemolysis (IC_{50})[a]	MIC (diffusion assay)[b]	MIC (dilution assay)	Therapeutic index[b]	Therapeutic index[c]
Pin2	3.3 (1.9–4.6)	37.5	12.5	0.09	0.26
Pin2 [P14V]	6.4 (0.0–13.2)	NA	12.5	–	0.51
Pin2 [P14GV]	9.3 (0.1–18.5)	300	12.5	0.03	0.74
Pin2 [P14VG]	8.8 (2.2–15.4)	300	12.5	0.03	0.70
Pin2 [P14GVG]	11.5 (8.4–14.6)	37.5	12.5	0.3	0.92

NA No antimicrobial activity

[a] Mean and 95% confidence intervals

[b] Therapeutic index represents IC_{50} (hemolysis)/MIC (diffusion assay)

[c] Therapeutic index represents IC_{50} (hemolysis)/MIC (dilution assay)

bacteria used under the agar diffusion assay. Such result may be related to the hydrophobicity of Pin2 [V]. The unexpected lack of activity of Pin 2 [V] and the low activity of Pin2 [VG] and [GV] may be due to its limited diffusion through the agar media primarily because of the hydrophobic nature of such peptide. Similar results were observed for Nisin an antimicrobial peptide produced by *Lactococcus lactis* (Chandrapati and O'Sullivan 1998). Usually, a major limitation of plate diffusion assays is that antibiotic diffusion through agar media is proportional to its concentration only over a limited linear range, and such antibiotic levels should be restricted to this range. Beyond this range, the zone diameters cannot be accurately related directly to the concentration of such antibiotics (Chandrapati and O'Sullivan 1998). In the liquid culture medium all five peptides kept their antimicrobial properties. There were no significant differences ($p < 0.05$) in the biological activity among all five peptides towards *S. aureus* (Fig. 3a). The MIC of Pin2 and Pin2 variants in the liquid culture medium was similar because they all had the same value; so, this data is valuable because one of the main potential applications of antimicrobial peptides is on topical infections, which develop in semi-solid environments such skin wounds where peptide diffusion plays an important role. Therefore, for topical clinical applications, the MIC values of plate diffusion assays offer advantages over that of MIC values in the liquid culture medium.

Concerning the hemolytic activities, the peptide variant P14 [GVG] had the lowest hemolytic activity of all five antimicrobial peptides. Such results are probably related to the composition of the cellular membrane. Eukaryotic membrane cells are more enriched in neutral phospholipids and cholesterol than bacterial membranes, which had a significant proportion of acidic phospholipids such phosphatidic acids.

The therapeutic index defined as the relationship between the hemolysis and the MIC values of all antimicrobial peptides was better for the variant Pin2 [GVG] (Table 3). Therefore, modifications of the proline residue for other amino acid motifs could be one way to improve the therapeutic index of highly hemolytic antimicrobial peptides.

Structures involving two helices separated by a flexible hinge region or a kink in the chain associated with a proline were reported for melittin (Bazzo et al. 1988), caerin 1.1 (Wong et al. 1997), sarcotoxin IA (Iwai et al. 1993), or cecropin A (Holak et al. 1988). Contrary to such antimicrobial peptides, Pin2 has a strongly basic C-terminal linked to a neutral N-terminal by a rigid proline link. The overall structure deduced by NMR for Pin2 is also two amphipathic segments joined by the proline hinge (Corzo et al. 2001). Therefore, the purpose of this work was to replace such proline for a more flexible hinge such GV, VG or GVG similar to the flexible Gly-Val and Gly-Pro hinges found in other natural antimicrobial peptides such oxypinins, ponericins and cecropins. Here, we found that the proline is an essential residue in this peptide, but it can be substituted successfully with amino acid residues that maintain the hydrophobic/hydrophilic characteristics of the peptide as well as its secondary structure keeping the range in antimicrobial activity and reducing its hemolytic properties compared with the parental Pin2 antimicrobial peptide.

Acknowledgments This work was supported by grants from CONACyT 83962/2007 to E.V., and CONACyT 49773/24968 to G.C. The mass spectrometry determination conducted by Dr. Fernando Zamudio is greatly acknowledged. The authors also acknowledge QBP Ma. Rocio Patiño Maya from Instituto de Química–UNAM for CD spectra data acquisition. Alexis Rodriguez is recipient of a PhD scholarship (#199738) from CONACyT.

Conflict of interest statement The authors declare that they have no conflict of interest.

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