Antimicrobial peptides from plants: stabilization of the γ core of a tomato defensin by intramolecular disulfide bond.

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Abstract

Cysteine-containing antimicrobial peptides of diverse phylogeny share a common structural signature, the γ core, characterized by a strong polarization of charges in two antiparallel β sheets. In this work we analyzed peptides derived from the tomato defensin SolyC07g007760 corresponding to the protein γ core and demonstrated that cyclization of the peptides, which results in segregation of positive charges to the turn region, produces peptides very active against Gram negative bacteria, such as *Salmonella enterica* and *Helicobacter pylori*. Interestingly these peptides show very low hemolytic activity and thus represent a scaffold for the design of new antimicrobial peptides.

Keywords: tomato defensin, cyclization, antimicrobial

Introduction

A tremendous effort has been recently devoted to the discovery of biologically active peptides from natural sources.[1-3] Plants produce a wide variety of antimicrobial peptides and proteins showing activity against either plant or human pathogens.[4] Many of these, such as thionins and knottins are rich in cysteines and are stabilized by a number of disulfide bonds.[5, 6] Interestingly some of the structural features displayed by peptides produced in plants are well conserved also within proteins produced by evolutionarily diverse organisms and, in fact, the existence of multidimensional signatures for antimicrobial peptides has been hypothesized.[7-9] The γ core motif has recently been indicated as a three dimensional signature for antimicrobial peptides or proteins and, generally, as a feature shown by membrane-active proteins. In particular, the γ core motif, characterized by a conserved CXG sequence and two antiparallel β sheets, is common to host defence polypeptides showing multiple disulfide bonds such as toxins, kinocidins and thionins.[10, 11] This structural motif can be generated by different amino acid sequences and can exist in the dextromeric or levomeric form, depending on whether it is derived by the amino acid forward or reverse sequence (Figure 1). In all cases γ core motifs show polarization of positive charge and segregation of hydrophobic amino acids. There are some cases in which the only γ core is sufficient for the peptide antimicrobial activity, as observed for protegrins, θ defensin RTD-1, and other cases in which the γ core represents the scaffold around which helices and sheets packs, as for kinocidins.[12, 13]

In a recent paper we identified, after a bioinformatics analysis of the tomato genome, the tomato defensin SolyC07g007760 (iTAG v.2.3) and demonstrated that a synthetic peptide (SolyC) corresponding to its γ core exerts antimicrobial activity against both Gram positive and Gram negative bacteria, being particularly active against different strains of the Gram negative bacterium *H. pylori*.[14, 15] Interestingly the peptide SolyC was able to discriminate between

pathogens and probiotic bacteria, killing only the minority of the probiotic tested. In addition, SolyC down-regulated the pro-inflammatory cytokines to an extent comparable to the known anti-inflammatory drug acetyl salicylic acid , did not cause red blood cell hemolysis and was not toxic toward eukaryotic cells.

SolyC is a peptide composed by 17 amino acids and contains three cysteines in positions 6, 13 and 15. The results described so far were obtained employing the peptide in its linear form. The presence of three cysteines which might be subjected to spontaneous oxidation and the observation that in typical γ cores those cysteines are involved in disulfide bonds urged us to investigate how formation of disulfide bonds in a controlled fashion could affect the secondary structure and the antimicrobial activity of the peptide. Two peptides in which Cys13 or Cys15 was mutated into a serine were obtained in the linear and oxidized form. Disulfide bonds were formed between C6 and C13 (SolyC1) or C6 and C15 (SolyC2); in both cases one cysteine was missing. Truncated peptides, starting from the conserved GXC motif were also obtained in the linear and oxidized form. The secondary structure of all peptides was analyzed by Circular Dichroism; the antimicrobial activity of the peptides was tested against Gram positive and Gram negative bacteria. All the oxidized peptides are highly active against Gram negative bacteria; the linear peptide SolyC2 shows antimicrobial activity against Gram negative bacteria comparable to that of the reference peptide SolyC, while linear SolyC1 exhibits an antimicrobial activity against Gram negative bacteria at higher concentrations. The activity of modified peptides against Gram positive bacteria was found in all cases lower as compared to that of the reference peptide. These results suggest that the interaction of the peptides with the membranes of Gram negative bacteria is stronger when the positive charges are exposed and this likely occurs when the peptides are in the oxidized form, in which the antiparallel β sheets are constrained by the disulfide bond.

Materials and methods

Reagents

The amino acids used for the peptide synthesis, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ser(OtBu)-OH, Fmoc-Thr(tBu)-OH , the Rink amide MBHA resin and the activators N-Hydroxybenzotriazole (HOBT) and O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were purchased from Novabiochem (Gibbstown, NJ, USA). Acetonitrile (ACN) was from Reidel-deHaën (Seelze, Germany) and dry N,N-dimethylformamide (DMF) from LabScan (Dublin, Ireland). All other reagents were from Sigma Aldrich (Milan, Italy). LC-MS analyses were performed on a LC-MS Thermo Finnigan with an electrospray source (MSQ) on a Phenomenex Jupiter 4µ Proteo (50x4.6 mm) column for all peptides except for SolyC-t which was analysed on a Jupiter 4µ Proteo (150x4.6 mm) column. Purification was carried out on a Onyx monolithic semi-prep C18 (100x10mm) column.

Synthesis

Peptides were synthesised on solid phase by Fmoc chemistry on the MBHA (0.54 mmol/g) resin by consecutive deprotection, coupling and capping cycles.[16] Deprotection: 30% piperidine in DMF, 5 minutes (2x). Coupling: 2.5 equivalents of amino acid + 2.49 equivalent of HOBT/HBTU (0.45 M in DMF/DMSO 75/25 v/v) + 3.5 equivalents NMM, 40 minutes. Capping: acetic anhydride/DIPEA/DMF 15/15/70 v/v/v, 5 minutes.

Peptides were cleaved off the resin and deprotected by treatment of the resin with a solution of TFA/TIS/EDT/H_2O 94/1/2.5/2.5 v/v/v/v, 3h, rt. TFA was concentrated and peptides were precipitated in cold ethylic ether. Analysis of the crudes was performed by LC-MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 70% in 15 minutes. Purification was

performed by semipreparative RP-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 70% in 15 minutes.

SolyC (Da): Calculated for 1994.32: $[M+2H]^{2+}$: 998.16; $[M+3H]^{3+}$: 665.77; Found: $[M+2H]^{2+}$: 997.55; $[M+3H]^{3+}$: 665.64;

Retention Time: 5.40 min

SolyC-t (Da): Calculated for 1703.01: $[M+2H]^{2+}$: 853.50; $[M+3H]^{3+}$: 568.67; Found: $[M+1H]^{1+}$: 1702.58; $[M+2H]^{2+}$: 852.29; $[M+3H]^{3+}$: 568.52;

Retention Time: 12.0 min

SolyC1 (Da): Calculated for 1978.26: $[M+2H]^{2+}$: 990.13; $[M+3H]^{3+}$: 660.42; Found: $[M+2H]^{2+}$: 990,13; $[M+3H]^{3+}$: 660.41;

Retention Time: 5.12 min

SolyC1-t (Da) Calculated for 1686.95: $[M+2H]^{2+}$: 844.47; $[M+3H]^{3+}$: 563.31; Found: $[M+2H]^{2+}$: 844.40; $[M+3H]^{3+}$: 563.21;

Retention Time: 5.12 min

SolyC2 (Da): Calculated for 1978.26 $[M+2H]^{2+}$: 990.13; $[M+3H]^{3+}$: 660.42; Found: $[M+2H]^{2+}$: 989.90; $[M+3H]^{3+}$: 660.21

Retention Time: 5.33 min

SolyC2-t (Da): Calculated for 1686.95: $[M+2H]^{2+}$: 844.47; $[M+3H]^{3+}$: 563.31; Found: $[M+2H]^{2+}$: 844.03; $[M+3H]^{3+}$: 563.23;

Retention Time: 4.93 min

Oxidation reaction

Disulfide-bridged analogues were obtained from the purified linear peptides after an oxidation reaction. Linear peptides were dissolved in a mixture of H_2O/acetic acid (95/5, v/v) at a final concentration of 2.5mM (9mL total volume); pH was adjusted to 6 with ammonium hydrogen carbonate and then DMSO (1 mL) was added. The reaction was complete after 24h. Analysis of the crudes was performed by LC-MS using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 70% in 15 minutes. Purification was performed by semipreparative RP-HPLC using a gradient of acetonitrile (0.1% TFA) in water (0.1% TFA) from 5 to 70% in 15 minutes.

SolyC1_ox (Da): Calculated for 1976.26: $[M+2H]^{2+}$: 989.13; $[M+3H]^{3+}$: 659.75; Found: $[M+2H]^{2+}$: 988.70; $[M+3H]^{3+}$: 659.60;

Retention Time: 5.35 min

SolyC1-t-ox (Da): Calculated for 1684.94: $[M+2H]^{2+}$: 843.47; $[M+3H]^{3+}$: 562.64; Found: $[M+2H]^{2+}$: 843.23; $[M+3H]^{3+}$: 562.53;

Retention Time: 4.96 min

Solyc2-ox (Da): Calculated for 1976.26: $[M+2H]^{2+}$: 989.13; $[M+3H]^{3+}$: 659.75; Found: $[M+2H]^{2+}$: 988.60; $[M+3H]^{3+}$: 659.60;

Retention Time: 5.10 min

SolyC2-t-ox (Da): Calculated for 1684.94: $[M+2H]^{2+}$: 843.47; $[M+3H]^{3+}$: 562.64; Found: $[M+2H]^{2+}$: 843.26; $[M+3H]^{3+}$: 562.50;

Retention Time: 5.15 min

Bacteria

Bacterial isolates were from patients hospitalized at the Medical School of the University of Naples "Federico II" and at the "Villa Betania" hospital (Naples, Italy). Species identification was carried out by PCR.[17-21] Bacteria were grown at 37°C in TSB or LB medium, harvested in the exponential phase (OD 600 nm 0.6-0.8), centrifuged (10 min at 8x103 g) and resuspended in Muller Hinton broth at the concentration of ~10^5 CFU/ml. The resistance/susceptibility of the different strains used in this study to conventional antibiotics were also determined using the disk diffusion method on Mueller-Hinton agar according to the NCCLS guidelines 2002 (data not shown)

Antibacterial and haemolytic activity

Bacteria were distributed in triplicate into plates (60 µl/well), mixed with dilutions of the peptides (5 – 100 µg/ml; 40 µl/well) and incubated at 37°C for 20 h. The minimal concentration of peptides causing 100% growth inhibition (MIC100), was determined by measuring the absorbance at 600 nm (Biorad microplate reader model 680, CA).[22] The test was performed in triplicate. The haemolytic activity of the peptides was tested using mouse red blood cells. The haemolytic activity was measured according to the formula OD peptide - OD negative control/OD positive control - OD negative control x 100 where the negative control (0% haemolysis) was represented by erythrocytes suspended in saline and the positive control (100% haemolysis) was represented by the erythrocytes lysed with 1% triton X100 .[22]

The LC50 value relative to the SolyC was calculated as described.[23]

Circular dichroism

Circular dichroism (CD) spectra were recorded at 25° C using a 1 cm quartz cell with the Jasco-810 spectropolarimeter using a 260 – 200 nm measurement range, 100 nm/min scanning speed, 1nm bandwidth, 4 sec response time, 0.5 nm data pitch.

Peptides concentration for CD measurement was 25 μM. CD spectra were registered in 10 mM sodium phosphate buffer, pH 7 and in 10 mM sodium phosphate, 20 mM SDS buffer, pH 7.

Results

Peptide design and synthesis

The SolyC peptide (Table 1) contains the γ core sequence of the tomato defensin Solyc07g007760 and shows strong similarity in the primary sequence with the levomeric form of porcine protegrin PG-1. In PG-1 four cysteines form two disulfide bonds, one involving C6 and C15 and the other C8 and C13, which force the peptide to fold into two antiparallel beta sheets; the oxidation of cysteines induces the formation of a turn characterized by a strong concentration of positive charges.[24, 25]

SolyC contains only three cysteines, therefore, in order to induce the peptide to assume a PG-1 like structure, only one disulfide bond can be formed. We designed two peptides with a different disulfide pattern, with the aim to explore the effect of cyclization on the antimicrobial activity of the peptide, to understand whether there is a preference for the formation of one of the two disulfides and also whether there is a difference in the activity of the peptides with cycles of different size (Table 1). Since in γ cores cysteines contained in sequences CXC are rarely connected

by disulfide bonds[26], we explored only two of the possible combinations of disulfides, namely C6-C13 (SolyC1) and C6-C15 (SolyC2) and mutated the cysteine not involved in the disulfide into a serine. In order to investigate the effect of reducing the hydrophobicity of the peptide, we also obtained truncated peptides, lacking three amino acids at the N-terminus. In the truncated peptides the residues which characterize the γ core motif GXC were conserved (Table 1). HPLC retention times for truncated peptides are similar or lower as compared to those of the full length peptides, as expected comparing the mean hydrophobicity calculated for each peptide (Table 1).[27]

All peptides were obtained in the linear and oxidized form. Oxidation was carried out incubating linear peptides with DMSO (10%) at pH 6.0. LC-MS analysis of the peptides, showing for the majority of the peptides a decrease of the retention time and a reduction of 2 units in the mass, confirmed that the reaction occurred (Figure 2).

Secondary structure studies

Linear and oxidized peptides were analyzed by Circular Dichroism at a 50μM concentration in Phosphate buffer, pH7. Peptides are mostly in an unordered form; spectra for the linear peptides, in fact, show one minimum around 204 nm and a very shallow minimum around 230 nm; upon oxidation the minimum shifts toward 207 nm. Spectra recorded in the presence of SDS 30 mM are in most cases very similar to those recorded in buffer, but more intense (Figure 3).

Antimicrobial activity test

The antimicrobial activity of the peptides was tested against the Gram positive bacteria *S. aureus*, *S. epidermidis*, *L. monocytogenes* and against the Gram negative bacteria *H.pylori* and *S. enterica*. (Table 2) For the standard peptide SolyC, truncation results in loss of activity against the Gram positive *Staphilococci*, while no difference was found when the peptides SolyC and SolyC-t were

tested against Gram negative bacteria. When tested against Gram positive bacteria, the analogues containing two cysteines (SolyC1 and SolyC2) show a reduced antimicrobial ability as compared to the reference peptide SolyC; MIC values are in all cases higher than that of SolyC and there is no large difference between the activity of linear and oxidized peptides. Peptides are in general more active against Gram negative bacteria; linear peptides show lower MIC in the full length version as compared to the truncated form. Noteworthy SolyC2, the peptide having the disulfide between C6 and C15, shows activity identical to that of the reference peptide. Oxidation results in an increase of the antimicrobial activity of the modified peptides against Gram negative bacteria, either in the extended or in the truncated form: all oxidized peptides exhibit activity identical or comparable to that of the reference peptide.

The hemolytic activity of the peptides was tested for all peptides at several concentrations (Figure 4). Up to 90μg/mL all peptides have hemolytic activity below 30%. At low concentrations all long linear peptides and the oxidized form of SolyC1 show hemolytic activity comparable to that of truncated peptides. Interestingly SolyC2 has in the long oxidized form (SolyC2-ox) very low hemolytic activity, comparable to that of the standard peptide.

LC50 measured for all peptides was found lower for the peptides containing only two cysteines as compared to SolyC (Table 3). The lowest LC50 value was observed for SolyC2 in the oxidized form (SolyC2-ox).

Discussion

Peptides containing a γ core motif and in particular analogues of protegrins have widely been studied as they are capable to exert antimicrobial activity even at high salt concentrations, unlike other antimicrobial peptides which form sheets such as β defensins.[28, 29] Interestingly, the PG-1

analogue IB-367 has been tested in phase II clinical trials for the treatment of oral mucositis and PG-1 based peptidomimetics were found to be active at nanomolar concentrations against *Pseudomonas aeruginosa*.[30, 31] It has been reported that the stabilization of the hairpin is necessary for the activity: PG-1 analogues lacking cysteines, in which the hairpin is stabilized by a tryptophan zipper motif, possess antimicrobial activity comparable to PG-1.[32] In PG-1 the number of disulfide bonds and their position affect the activity; analogues of PG-1 with a single disulfide bond possess antimicrobial activity comparable to PG-1 in the linear form.

The γ core of the tomato defensin family, to which SolyC peptides belong, has a strong similarity in the amino acid sequence with the γ core of other plant defensins as the Medicago sativa defensin 4 (MtDef4), and also with the levomeric form of PG-1.[9, 33] The three-dimensional structures of plant defensins, which are composed of about 50-60 amino acids, are very complex and cysteines belonging to the γ core form disulfide bonds with cysteines located outside of the γ core.[34-36] In self-consistent γ cores as PG-1 and RTD-1, instead, the beta-hairpin structure is stabilized by two disulfide bonds between cysteines inside the γ core. SolyC is a fragment of a tomato defensin and, as most of the plant defensins, has a very conserved GXCRG motif within the γ core with three cysteins. It is reasonable to think that SolyC has a structural organization similar to that of plant defensin in which cysteines in the γ core are not connected between them. As we have demonstrated in a previous paper that the linear fragment SolyC possess high antimicrobial activity, we now explored the possibility of stabilizing the peptide structure introducing in a controlled fashion one disulfide bond, and evaluated the effects of cyclization on the peptide antimicrobial activity and secondary structure. Furthermore similar studies were carried out on truncated peptides.

In SolyC cysteins are located in position i, i+7 and i+9; peptides with disulfide bonds between Cys i –i+7 (SolyC1) and i-i+9 (SolyC2) were obtained. The secondary structure of all peptides was

analyzed by CD in phosphate buffer and in phosphate buffer containing SDS: both linear and oxidized peptides seems to be in an unordered form (Figure 3). All the analogues are more active against Gram negative than against Gram positive bacteria; these results are in line to what reported for PG-1 and PG-1 analogues with a single disulfide bond.[28] Interestingly, cyclic peptides are generally more active than linear ones against Gram negative bacteria. Cyclization induce a strong polarization of the peptides, confining the positive charge in the turn and the hydrophobic residues on the "tails", and reasonably allows the peptide to interact with the bacterial LPS, neutralizing and permeating it. (Figure 5) Recent studies aimed to characterize the interaction of rationally designed peptides with the LPS demonstrate that amphipatic peptides are active antimicrobials as they assume the structure of a β boomerang upon interaction with LPS, exerting their activity by disgregating it.[37] These peptides are mimics of the outer membrane, as they possess a polar head and hydrophobic tails and easily intercalate into LPS. The SolyC cyclic analogues are already in the active conformation with the segregation of charges and therefore it is likely that they exert their activity as the β boomerang peptides do.

SolyC2 in the full length and truncated version shows antimicrobial activity identical to that of the reference peptide SolyC. These result may be interpreted hypothesizing that, in the experimental conditions employed for the activity test, SolyC spontaneously oxidizes and the formation of the disulfide bond between Cys in position i and i+9 is favored. As in SolyC2 only the disulfide bridge between Cys i and i+9 can be formed, it is reasonable to think that the linear peptide spontaneously converts into the oxidized form which corresponds also to the active form. This hypothesis is consistent with the observation that full length SolyC2 in the linear and oxidized form has the same antimicrobial activity as the reference SolyC. Interestingly in SolyC2 the cysteines involved in the disulfide bond are located in positions identical to external cysteines of PG-1, reproducing the cyclization pattern of the highly active "bullet" peptide proposed by Harwig.[28]

The observation that there is no difference in the activity against Gram negative bacteria of cyclic SolyC1 and SolyC2 derivatives, which differ in the number of amino acids included in the cycle, suggests that the charge segregation and not the dimension of the cycle is crucial for activity.

All peptides at concentrations up to threefold that of MIC show very low hemolytic activity, indicating that these peptides are selective for bacterial outer membrane. The lowest percentage of hemolytic activity was found for the oxidized SolyC2. LC50 calculated for all peptides on murine red blood cells is lower for SolyC2, confirming the selectivity for negatively charged membrane (as the bacterial LPS) versus zwitterioninc membranes (as the erythrocytes).

In conclusion, we have demonstrated that isolated γ cores of plant defensins possess strong antimicrobial activity against Gram negative bacteria; formation of an intramolecular disulfide bond stabilizes the peptides in the "active" conformation. We found that the peptide SolyC2, which reproduces the disulfide bond pattern observed in PG-1, shows an high selectivity towards bacterial outer membrane, showing very low hemolytic activity at concentrations which are threefold the MIC. These results encourage future studies on the antimicrobial activity of isolated γ cores peptides from plants, due to their strength and specificity of action.

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Figure

Figure 1: Schematic representation of the gamma core isoforms.

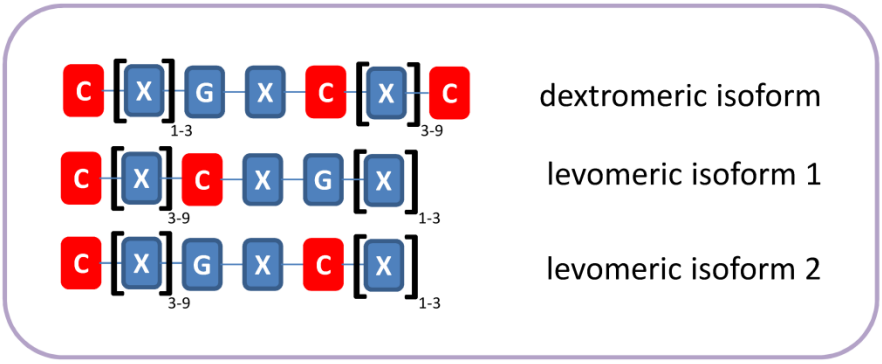


Figure 2: Comparison of the LC profiles for SolyC2 in the linear (A) and oxidized form (B). Zoom between 4 and 6 minutes of the LC run.

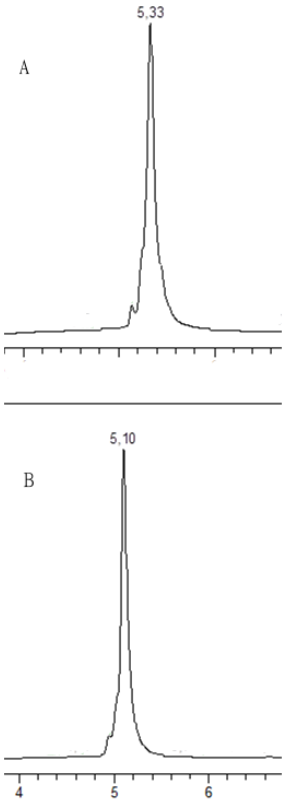


Figure 3: CD spectra of SolyC2 (Blue) and SolyC2-ox (red) (50µM) recorded in 10mM Phosphate buffer pH7 (continuous line) and in 10mM Phosphate buffer, 30mM SDS pH7 (dotted lines).

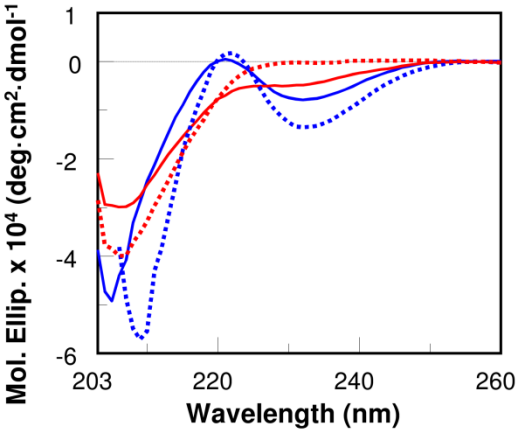


Figure 4: Hemolytic activity of the peptides measured at different concentrations.

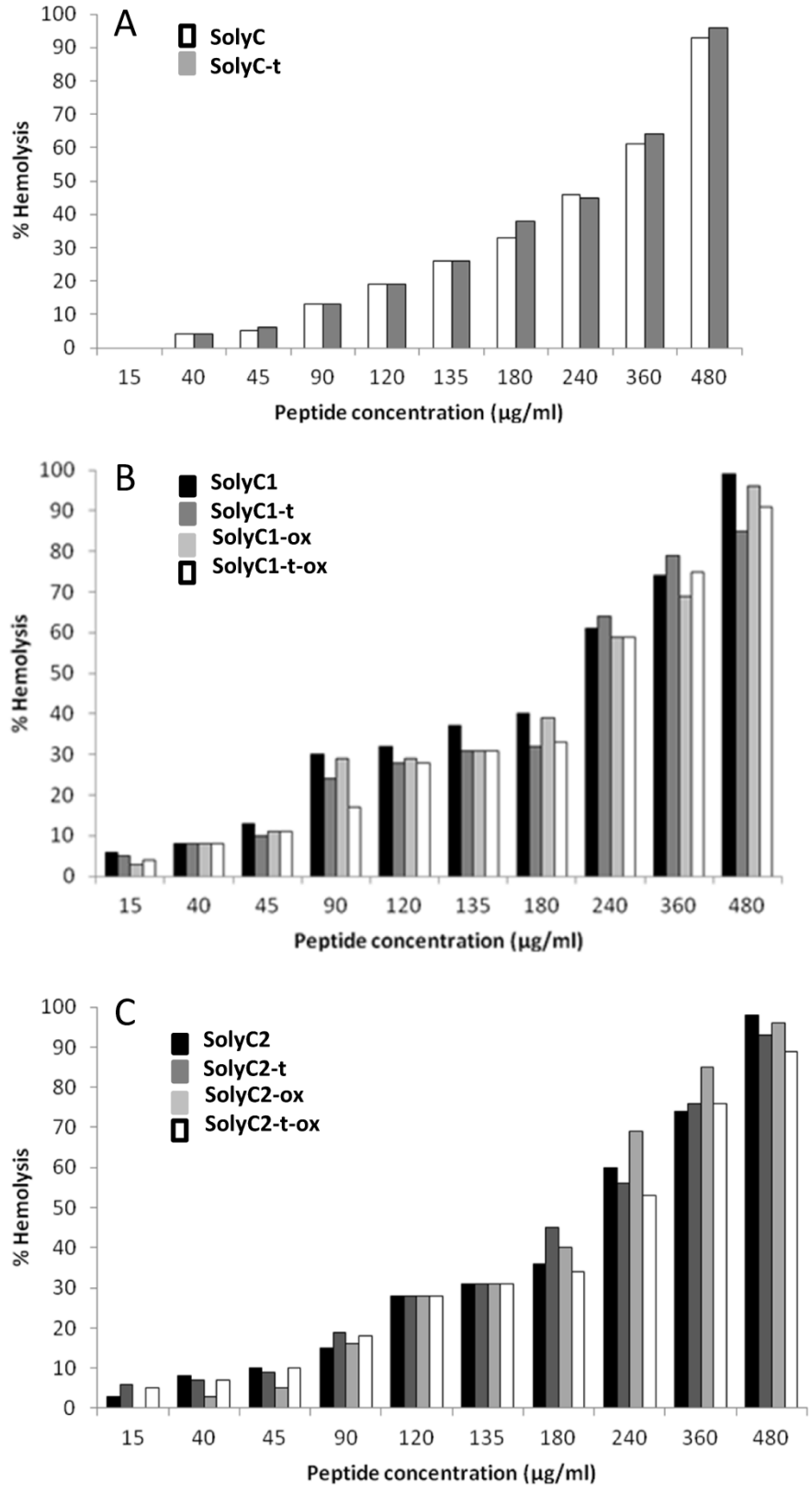


Figure 5: Schematic representation of the peptide SolyC2-ox; in red the positively charged amino acids in the turn, in yellow the cysteines involved in the disulfide bond and in green the cysteine residue mutated into serine.

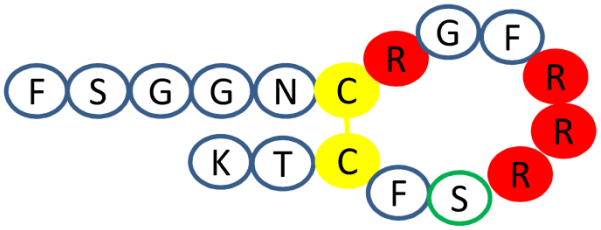


Table1: Peptide sequences, names, reference number and mean hydrophobicity

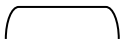
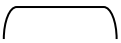
Peptide sequence	Name	Number	Mean Hydrophobicity
FSGGNCRGFRRRCFCTK	SolyC	1	-0.71
GNCRGFRRRCFCTK	SolyC -t	2	-0.98
FSGGNCRGFRRRCFSTK	SolyC1	3	-0.91
GNCRGFRRRCFSTK	SolyC1-t	4	-1.22
FSGGNCRGFRRRSFCTK	SolyC2	5	-0.91
GNCRGFRRRSFCTK	SolyC2-t	6	-1.22
 FSGGNCRGFRRRCFSTK	SolyC1-ox	7	--0.91
 GNCRGFRRRCFSTK	SolyC1-t-ox	8	-1.22
 FSGGNCRGFRRRSFCTK	SolyC2-ox	9	-0.91
 GNCRGFRRRSFCTK	SolyC2-t-ox	10	-1.22

Table2: Antimicrobial activity of the peptides against Gram positive and Gram negative bacteria

STRAINS	**MIC_{100} (μg/ml)**									
	Peptide number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Staphylococcus aureus	40	50	50	50	80	80	80	50	100	80
Staphylococcus epidermidis	40	80	100	100	100	100	100	100	100	100
Listeria monocytogenes	80	80	80	80	80	80	80	80	80	80
Salmonella serovar paratyphi B	15	15	40	80	15	40	15	15	15	20
Helicobacter pylori	15	15	15	15	15	20	15	15	20	20

Table3: LC50 for the SolyC and SolyC analogues

	Peptide number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
LC 50 (µg/ml)	275.2	266.7	212.3	228.5	226.5	213.0	229.6	231.0	197.4	235.1