A library of linear undecapeptides with bactericidal activity against phytopathogenic bacteria

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1. Introduction

Bacterial diseases of plants are currently one of the major factors limiting worldwide crop production [1], and their control is mainly based on copper compounds and antibiotics [30,52]. Although antibiotics are highly efficient, they are not authorized in several countries and resistance has been developed on plant pathogens. Therefore, there is a need for new compounds with low environmental impact, broad spectrum of activity, reasonable bacterial selectivity, and low eukaryotic cytotoxicity. Several antimicrobial peptides (AMPs) fit these features, and a great deal of scientific effort has been invested in studying their application in human, veterinary and plant disease control [24,25,33,55].

AMPs are components of the innate immune system found in a wide range of organisms, including plants, insects,
amphibians and humans [12–14,18,19,37,54]. AMPs are also secreted by microorganisms and include small bacteriocins, fungal defensins, peptaibols, cyclopeptides, and pseudopeptides [33,43]. In general, AMPs have a net positive charge and the ability to assume an amphipathic structure, which confer an electrostatic affinity to the negatively charged outer leaflet of the microbial membrane and enable the integration of the peptide into the hydrophobic core of the cell membrane [6,9,11,23,44,49,53]. However, there is increasing evidence that apart from membrane damage, other mechanisms may be involved including intracellular targets [11,25,53]. The diversity of mechanisms of action has led to the belief that the frequency of resistance emergence to AMPs should be low [11,40]. Moreover, most AMPs are selective for bacteria or eukaryotic cells that may be explained by the cationic nature of these peptides and the different net charge of the anionic lipids in bacteria and mammalian cells [11,22,31,53].

Natural AMPs usually have long amino acid sequences, display poor bioavailability and are prone to protease degradation. To overcome these limitations, research has been carried out in order to find shorter and more stable peptides, while maintaining or increasing the activity with a low cytotoxicity [3,16,20,25,55]. However, the process involved in the development of lead candidates is time-consuming and limited by the number of individual compounds that can be synthesized. Combinatorial chemistry is an efficient method for generating large number of compounds in a short period of time. In fact, it has been successfully used for identifying and improving peptides [7,8,42,47], and synthetic libraries have been screened to find linear and cyclic peptides with activity against selected phytopathogenic bacteria and fungi [28,29,32].

Our current research is oriented to the identification of short synthetic peptides with specific activity against economically important plant pathogenic bacteria [15,34,35]. Our efforts are mainly focused on E. amylovora, the causal agent of fire blight, a severe disease that affects rosaceous plants and causes great economic losses in pear, apple and ornamental plant production [15,50,51]. Moreover, we are also interested in developing new control methods against Xanthomonas axonopodis pv. vesicatoria, causing bacterial spot of tomato and pepper, and Pseudomonas syringae pv. syringae, causing several blight diseases [34,35]. To date, apart from copper bactericides and the antibiotics streptomycin and tetracycline registered in certain countries, it has not been described any effective method to treat these plant diseases. Moreover, the emergence of strains resistant to streptomycin that decreases the efficacy of disease control, the environmental impact of antibiotics, and consumer concerns about residues in food have favored the development of alternative or complementary methods for fire blight management [27,46].

Recently, we have identified cecropin A-melittin hybrid undecapeptides which inhibited in vitro growth of the above plant pathogenic bacteria [5,17]. One of the best peptides, H-K’KLFKKLKFL’L-NH2 (BP76), also showed minimized cytotoxicity and low susceptibility to protease degradation, being a good candidate to be further tested in vivo. However, since in vitro results do not necessarily reflect the efficacy of peptides in vivo, a large number of peptides with potential in vitro activity must be prepared in order to successfully find in vivo active compounds. With the aim of obtaining a set of BP76 analogues with in vitro activity, a library could be prepared including variations at positions 1 and 10 as well as at the N-terminus, which resulted to influence the antibacterial and hemolytic activities of BP76 [17].

In the present study, a 125-member library was synthesized using a combinatorial chemistry approach and screened for in vitro growth inhibition of E. amylovora, X. axonopodis pv. vesicatoria, and P. syringae pv. syringae. The most effective compounds were evaluated for hemolytic activity, bactericidal effect, proteolytic susceptibility and inhibitory activity in an in vivo model system based on E. amylovora infection in detached apple and pear flowers.

2. Material and methods

2.1. Materials

The 9-fluorenylmethoxycarbonyl (Fmoc) acid derivatives, reagents, and solvents used in the peptide synthesis were obtained from Senn Chemicals International (Gentilly, France). Fmoc-Rink-4-methylbenzhydrylamine (MBHA) resin (0.64 mmol/g) was purchased from Novabiochem (Darmstadt, Germany). Trifluoroacetic acid (TFA), N-methyl-2-pyrrolidinone (NMP), and triisopropylsilane (TIS) were from Sigma–Aldrich Corporation (Madrid, Spain). Piperidine, N,N-diisopropylpyrrolidylethylamine (DIEA), pyridine, benzyl bromide, acetic anhydride, benzoyl chloride and p-toluenesulphonyl chloride were purchased from Fluka (Buchs, Switzerland). Solvents for high-performance liquid chromatography (HPLC) were obtained from J.T. Baker (Deventer, Holland).

2.2. Synthesis of the peptide library

The peptide library was prepared manually by the solid-phase method using Fmoc-type chemistry, Fmoc-Rink-MBHA resin (0.64 mmol/g) as solid support, tert-butyloxycarbonyl side-chain protection for Lys and Trp, and couplings of the Fmoc-amino acids (3 equiv.) mediated by N-[1H-ben佐za-1-yl](dimethylaminomethylene)-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU) (3 equiv.) and DIEA (3 equiv.) in N,N-dimethylformamide (DMF) and monitored by ninhydrin test. Fmoc group was removed by treating the resin with a mixture of piperidine–DMF (3:7). Washings were performed with DMF.

The Fmoc-Rink-MBHA resin was placed into a plastic syringe fitted with a polypropylene frit to remove the Fmoc group and to subsequently couple Fmoc-Leu-OH. After Fmoc group removal, the resin was partitioned into five equal parts which were transferred into five plastic syringes. Each portion of resin was treated with one amino acid (Fmoc-Val-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(Boc)-OH, Fmoc-Phe-OH or Fmoc-Lys(Boc)-OH) under coupling conditions. Next coupling steps were accomplished by repeated cycles of Fmoc group removal and coupling. Once the residue at position 2 was coupled, each of the five peptidylresins was partitioned into five equal parts to which Fmoc-Trp(Boc)-OH, Fmoc-Phe-OH, Fmoc-Tyr(tBu)-OH,
Fmoc-Lys(Boc)-OH or Fmoc-Leu-OH was coupled. Each of the resulting 25 peptidylresins was partitioned again into 5 equal parts. After Fmoc removal, four of the five portions of resin obtained were then derivatized at the N-terminus. Acetylation was performed by treatment with acetic anhydride–pyridine–
CH₂Cl₂ (1:1:1). Tosylation was carried out by treatment with p-
toluenesulphonyl chloride (40 equiv.) and DIEA (80 equiv.) in
CH₂Cl₂–NMP (9:1). Benzoylation was performed by treatment with
benzoyl chloride (40 equiv.) and DIEA (80 equiv.) in CH₂Cl₂–NMP (9:1). Benzoylation was achieved by treatment with benzyl bromide (40 equiv.) and DIEA (80 equiv.) in CH₂Cl₂–NMP (9:1). The 125 resulting peptides were individually cleaved
from the resin with TFA–H₂O–TIS (95:2.5:2.5). Following TFA
evaporation and diethyl ether extraction, the crude peptides
were dissolved in H₂O, lyophilized and tested for purity on
HPLC. Except for the N-terminal benzylated peptides which
were obtained as a mixture of mono and dibenzylated
derivatives, all peptides synthesized showed >90% HPLC
purity. Electrospray ionization mass spectrometry was used
to confirm peptide identity.

Library members are denoted using brace numbers as
[R,X1,X10], which define the variations at each R, X1 and X10
positions.

2.3 Bacterial strains and growth conditions

The following plant pathogenic bacterial strains were used:
Erwinia amylovora PMV6076 (Institut National de la Recherche
Agronomique, Angers, France), Pseudomonas syringae pv.
syringae EPS94 (Institut de Tecnologia Agroalimentària, Uni-
versitat de Girona, Spain) and Xanthomonas axonopodis pv.
esicctoria 2133-2 (Instituto Valenciano de Investigaciones
Agrarias, Valencia, Spain). For testing the efficacy in in vivo
assays, E. amylovora EPS101 (Institut de Tecnologia Agroali-
mentària, Universitat de Girona, Spain) was used. All bacteria
were stored in Luria Bertani (LB) broth supplemented with
glycerol (20%) and maintained at −80 °C. E. amylovora and Pss
were scrapped from LB agar after growing for 24 h and Xav
after growing for 48 h at 25 °C. The cell material was
suspended in sterile water to obtain a suspension of
10⁶ CFU ml⁻¹. The original suspension was 10-fold serially
diluted in sterile distilled water to obtain suitable concentra-
tions when necessary.

2.4 Antibacterial activity

Lyophilized peptides were solubilized in sterile Milli-Q water
to a final concentration of 1000 μM and filter sterilized through
a 0.22-μm pore filter. For minimum inhibitory concentration
(MIC) assessment, dilutions of the synthetic peptides were
made to obtain a final concentration of 75, 50 and 25 μM.
Twenty microlitres of each dilution were mixed in a microtiter
plate well with 20 μl of the corresponding suspension of the
bacterial indicator, 160 μl of Trypticase Soy Broth (TSB)
(BioMérieux, France) to a total volume of 200 μl. Three
replicates for each strain, peptide and concentration were
used. Positive controls contained water instead of peptide and
negative controls contained peptides without bacterial sus-
pension. Microbial growth was automatically determined by
optical density measurement at 600 nm (Bioscreen C, Labsys-
tem, Helsinki, Finland). Microplates were incubated at 25 °C
with 20 s shaking before hourly absorbance measurement for
48 h. The experiment was repeated twice. The MIC was taken
as the lowest peptide concentration with no growth at the end
of the experiment.

2.5 Hemolytic activity

The hemolytic activity of the 54 more active peptides was
evaluated by determining hemoglobin release from erythrocyte
suspensions of fresh human blood (5%, v/v). Blood was
aseptically collected using a BD vacutainer K2E System with
EDTA (Belliver Industrial State, Plymouth, UK) and stored for
less than 2 h at 4 °C. Blood was centrifuged at 6000 × g for 5 min,
washed three times with TRIS buffer (10 mM TRIS, 150 mM
NaCl, pH 7.2) and diluted. Peptides were solubilized in TRIS
buffer to a final concentration of 500, 300 and 100 μM. Fifty
microliters of human red blood cells were mixed with 50 μl of
the peptide solution and incubated under continuous shaking
for 1 h at 37 °C. Then, the tubes were centrifuged at 3500 × g
for 10 min. Eighty microliters aliquots of the supernatant were
transferred to 100-well microplates (Bioscreen) and diluted
with 80 μl of Milli-Q water. Hemolysis was measured as the
absorbance at 540 nm with a Bioscreen plate reader. Complete
hemolysis was determined in TRIS buffer plus melittin (Sigma–
Aldrich Corporation, Madrid, Spain) as a positive control.
The percentage of hemolysis (h) was calculated using the equation:

\[ h = 100 \times \left(1 - \frac{O_{b} - O_{m}}{O_{b} - O_{o}}\right) \]

where Oₙ was the density for a
given peptide concentration, Oₚ for the buffer, and Oₘ for the
melittin positive control.

2.6 Bactericidal activity

The bactericidal activity was determined for peptides BP76,
BP77, BP100, BP125, and BP126. LB broth grown cultures of
E. amylovora were inoculated at 4 × 10⁶ CFU ml⁻¹ in fresh LB
broth containing a 5.0 μM concentration of the corresponding
peptide and incubated at 25 °C. Aliquots of 500 μl were
removed at 30 min time intervals during 3 h, 10-fold diluted,
and the dilutions were plated on LB agar plates. Colony-
forming units were counted after 48 h incubation at 25 °C.
Values of surviving cells were expressed as a percentage of
survival from the start of the experiment. Decimal reduction
times (D) were calculated as the reverse of the slope values
obtained from log₁₀ survival versus time plots.

2.7 Susceptibility to protease degradation

Digestion of [HI,L,V], BP77, BP81, BP100, BP125, and BP126 was
carried out by treating 50 μg/ml peptide with 1 μg/ml
proteinase K (Sigma–Aldrich Corporation, Madrid, Spain)
in 100 mM TRIS buffer, pH 7.6, at room temperature. The
peptide cleavage after 5, 10, 15, 30 and 45 min was monitored
by HPLC using a Kromasil (4.6 mm × 40 mm; 3.5 μm particle
size) C₁₈ reversed-phase column. Linear gradients of 0.1%
aqueous TFA and 0.1% TFA in CH₃CN were run from 0.98:0.02
to 0:1 over 7 min with UV detection at 220 nm. Digestion
was estimated as the percentage of degraded peptide calculated
from the decrease of the HPLC peak area of the native
peptide.
2.8. Activity of peptides against *E. amylovora* infection in apple and pear flowers

Efficacy of peptides BP76, BP77, BP100, BP125 and BP126 was determined using infection assays of *E. amylovora* in pear flowers of cultivar Doyenne du Comice and apple flowers of cultivar Fuji as previously described [15]. Plant material was collected from commercial orchards of Girona (Spain). Individual flowers were maintained with the cut peduncle submerged in 1 ml of a 10% sucrose solution in a single plastic Eppendorf vial of 1.5 ml. Vials containing flowers were placed in plastic tube racks [41]. Peptides were applied by delivering 10 μl of the peptide solution on the surface of the hypanthium of flowers. The treated material was incubated at room temperature during 1 h. Then, the hypanthium of flowers was inoculated with 10 μl of a suspension of *E. amylovora* EPS101 at 10° CFU ml⁻¹. The inoculated plant material was incubated at 23 °C and high relative humidity for 5 days. The experimental design consisted of three replicates of eight flowers. Non-treated controls inoculated only with the pathogen was included. Controls of phytotoxicity consisting of treated flowers with the peptides without pathogen inoculation were used. Infection severity levels were determined for each flower according to a scale of severity from 0 to 3 according to the symptoms observed: 0, no symptoms; 1, partial hypanthium necrosis; 2, total hypanthium necrosis; 3, necrosis progression through peduncle. The mean severity was calculated for each replicate. The effect of peptides on flower infection was determined using analysis of variance (ANOVA) with the general linear models (GLM) procedure of statistical analysis system (SAS) (Version 8.2, SAS Institute Inc., NC, USA). Means were separated using the Tukey’s test (P < 0.05).

3. Results

3.1. Design of the peptide library

The peptide library was designed based on the ideal α-helical wheel diagram of H–K¹KLFKKILKF¹³-NH₂ (BP76) and comprised 125 peptides (Fig. 1). We investigated the influence on the biological activity of residues at positions 1 and 10 by incorporating amino acids possessing various degrees of hydrophobicity and hydrophilicity. Thus, Lys, Tyr, Leu, Phe or Trp were incorporated at position 1, and Lys, Tyr, Val, Phe or Trp were introduced at position 10. Blocking of the N-terminus with an acetyl, tosyl, benzoyl or benzyl group was also studied.

3.2. Antibacterial activity

The peptide library was tested for in vitro growth inhibition of *E. amylovora*, X. axonopodis pv. vesicatoria, and P. syringae pv. syringae at 2.5, 5.0, and 7.5 μM (Fig. 2). Most peptides (94.4%) exhibited relevant antibacterial activities (MIC < 7.5 μM) against at least one pathogen, except for [H,K,K], [H,Y,V], [H,L,K], [Ac,L,K], [H,F,K], [H,F,V], and [H,W,V]. Xav was more sensitive to each of the peptides than the other two bacteria. Eighteen peptides displayed higher activity against Xav than BP76 (MIC < 2.5 μM) and eighty-four sequences were as active as BP76 (MIC of 2.5–5.0 μM). Complete inhibition of *E. amylovora* and *Pss* was observed at the same MIC range than BP76 (2.5–5.0 μM) for six and eight peptides, respectively. Moreover, 21 and 32 peptides exhibited MIC values of 5.0–7.5 μM against *E. amylovora* and *Pss*, respectively. BP52, BP66, BP81, BP100, and BP105 were the most active N-terminal non-derivatized peptides (Table 1). N-terminal derivatization decreased the antibacterial activity against *Pss* and *E. amylovora*, while it increased the activity against Xav. N-terminal acetylated, tosylated, benzoylated and benzylated peptides showed similar MIC values, being BP31, BP32, BP77, BP78, BP102, BP103, BP121, BP125 and BP126 the most active.

In general, a Lys at position 1 was associated with high activity (Fig. 2). However, the non-derivatized peptide bearing a Lys at positions 1 and 10, [H,K,K], was inactive. Its activity was improved by N-terminal derivatization. For non-derivatized peptides accommodating at position 1 residues other than Lys, best activities were observed when position 10 was occupied by an aromatic residue.

3.3. Hemolytic activity

The toxicity to eukaryotic cells of the most active peptides was determined as the ability to lyse erythrocytes in comparison to melittin. Percent hemolysis at 50, 150 and 250 μM is shown in Fig. 3. Table 1 shows hemolysis at 50 and 150 μM for selected peptides. Seventeen peptides displayed <15% hemolysis at 50 μM. The presence of a Lys at position 1 was associated with low hemolysis and N-terminal derivatization increased the hemolytic activity. In general, the acetylated peptides were the less hemolytic N-terminal derivatized peptides. Peptides bearing a Trp either at position 1 or 10 displayed the highest hemolysis.
Fig. 2 – MICs of the 125-member peptide library against *E. amylovora*, *P. syringae* pv. *syringae* and *X. axonopodis* pv. *vesicatoria*. Amino acids of different degree of hydrophylicity and hydrophobicity (X) at positions 1 and 10, and N-terminal derivatizations (R) were introduced in R-X1KLFKKILKX10L-NH2. Derivatization corresponds to acetylation (Ac), tosylation (Ts) and benzoylation (Bz). Solid black bars stand for MIC < 5.0 μM, striped bars for MIC of 5.0–7.5 μM, and shaded bars for MIC > 7.5 μM.

Fig. 3 – Hemolytic activity of selected peptides of the library at 50, 150 and 250 μM. Amino acids of different degree of hydrophylicity and hydrophobicity (X) at positions 1 and 10, and N-terminal derivatizations (R) were introduced in R-X1KLFKKILKX10L-NH2. Derivatization corresponds to acetylation (Ac), tosylation (Ts) and benzoylation (Bz). The absence of columns indicates that hemolytic activity was not determined. Solid black bars stand for hemolysis percentage ≤ 50%.
3.4. Bactericidal activity

The bactericidal activity of selected peptides of the library was evaluated by comparing the time course to kill mid-logarithmic-phase culture suspensions of *E. amylovora* (Fig. 4). Kinetics of cell death for most peptides, except for BP77, followed a typical logarithmic pattern. Peptide BP77 showed an initial fast killing stage of 30 min, followed by a slower rate. Peptides BP76 and BP100 were the most active and were able to kill 99% of cells within 30 min at 5.0 μM, whereas BP77, BP125 and BP126 had lower activity. Decimal reduction times (D) of the peptides studied ranged from 36 to 78 min at the concentration around the MIC, and were 36 min for BP100 and 40 min for BP76.

3.5. Susceptibility to protease degradation

The susceptibility of the peptides to proteolysis was studied by exposure to proteinase K and degradation was monitored by reverse-phase HPLC over time (Fig. 5). BP81, BP100 and BP126 displayed similar susceptibility to protease degradation than BP76 and after 45 min incubation only 50% degradation was observed. The peptides [H,L,V], BP77 and BP125 were more susceptible than BP76. Peptide [H,L,V] was completely degraded after exposure to protease.

3.6. In vivo activity

All the peptides tested significantly reduced the severity of infections caused by *E. amylovora* in apple and pear flowers at 100 and 200 μM, except for peptide BP77 at 100 μM (Fig. 6). The best peptide was BP100, reducing the severity of infection in apple flowers from 1.90 (non-treated control) to 0.93 at 100 μM and to 0.73 at 200 μM. The severity of infection in pear flowers decreased from 1.40 (non-treated control) to 0.37 at 100 μM and to 0.40 at 200 μM. Disease reduction was 63% in apple and 74% in pear flowers. These percentages were only slightly lower than those obtained with streptomycin. No visible signs of phytotoxicity in flowers were observed at the peptide concentrations assayed.

4. Discussion

Small AMPs have drawn attention due to their potential for application in plant disease control [2,23]. In a previous work, several cecropin A-melittin hybrid undecapeptides with antibacterial activity against *E. amylovora*, Xau and Pss have been identified [5,17]. In particular, BP76, consisting of H-KKLFKHKKILKFL-NH2, was active against these bacteria at similar concentrations than conventional antibiotics, displayed low cytotoxicity, and showed significant stability towards protease degradation. These promising results prompted us to design a combinatorial library using BP76 as lead structure to improve the antibacterial activity of this peptide and minimize the cytotoxicity, the susceptibility to protease degradation and to test the best peptides in a triple interaction model system based on *E. amylovora* infection on detached flowers.

In the design of AMPs, important parameters to be considered are charge and amphipathicity. It is generally accepted that when AMPs interact with phospholipid membranes, there is an initial electrostatic attraction involving positively charged amino acids and, afterwards, they adopt an amphipathic conformation that allows the insertion of their hydrophobic face into the membrane bilayer [6,9,11,23,44,49,53]. The amphipathic character of BP76 becomes evident when it is represented as an ideal α-helix by means of an Edmunson wheel plot. Accordingly, in order to maintain the ability of peptides derived from BP76 to interact with the bacterial membrane, the 125-member peptide library was designed based on this ideal α-helical wheel diagram. Amino
acids with various degrees of hydrophilicity and hydrophobicity were introduced at positions X1 and X10 because they are located at the interface. The influence of the N-terminus derivatization was also studied.

A 94.4% of the 125-member library showed potent activity against at least one of the plant pathogenic bacteria tested. MIC values below 7.5 μM were obtained for 113, 27 and 40 peptides against Xav, E. amylovora, and Pss, respectively. In fact, some of the synthesized peptides were as potent as the parent peptide BP76 and, remarkably, 18 peptides were more active than BP76 against Xav. These results are in good agreement with previous reports, which showed that Xav was more susceptible than Pss and E. amylovora to short cationic peptides [17,32]. This different level of susceptibility to peptides between bacteria can be attributed to differences in the components of membrane of the target microorganism, e.g. charge and lipid composition, which would influence rates of binding of cationic peptides to the membranes [26].

Our results confirm previous data on how subtle changes in a peptide sequence influence antimicrobial and hemolytic activity [29,32]. Sequence–activity relationship studies have shown that at least seven parameters are relevant to the biological activity of peptides: the size, the sequence, the degree of structuring, the charge, the overall hydrophobicity, the amphipathicity, and the respective widths of the hydrophobic and hydrophilic faces of the helix [49]. The accomplishment of these structural parameters is not stringently necessary for activity against gram-negative bacteria, but rather a good balance between them is required. Interestingly, it has been shown that a threshold of hydrophobicity and a net positive charge are sufficient to endow a peptide with antimicrobial activity [38].

The effect of charge on activity against the plant pathogenic bacteria tested was clearly observed. N-terminus derivatization and a Lys at position 1 were crucial for activity. N-Terminal derivatization led to an important decrease of the antibacterial activity against E. amylovora and Pss, but afforded peptides with identical or improved MIC values against Xav. This difference could be attributed to the characteristic membrane and cell envelope of each bacteria. High activity was observed for peptides bearing a Lys at position 1. However, an increase of the size of the hydrophilic face by incorporating...
a Lys at both positions 1 and 10, together with a non-
derivatized N-terminus, resulted in a peptide with a net charge
of +7 which was inactive. In this case, N-terminal derivatiza-
tion led to peptides with antibacterial activity. Similarly to the
majority of natural peptides, activity was observed for
sequences with a net charge ranging from +4 to +6 [21]. In
particular, the most active peptides had a net charge of +5 or
+6. Moreover, activity was favored when position 10 incorpo-
rated an aromatic residue. These data confirm preliminary
results indicating that a basic N-terminus and a hydrophobic
C-terminus are important for the antibacterial activity [17].
The most active peptides against the three plant pathogenic
bacteria studied followed these general structural require-
ments, i.e. BP31, BP52, BP66, BP77, BP78, BP81, BP100, BP102,
BP103, BP105, BP121, BP125, and BP126.
Toxicity to animal or plant cells is always a problem in
AMPs targeted to bacteria or fungi when the mechanism of
action is based on interaction with cell membranes. Cyto-
toxicity can be evaluated using animal or plant cell model
systems. We have used red blood cells instead of plant cells
because of the highly standardized methods available and
data can be compared straightforwardly with previous
reports. Several studies have shown that an increase in the
peptide hydrophobicity is related to an increase of cytotoxicity
[8,36]. This behavior has been attributed to the differences in
membrane lipid composition between bacterial and mamma-
lian cells. The absence of acidic phospholipids and presence of
sterols reduce the susceptibility of eukaryotic cells to many
cationic peptides [31]. Accordingly, we have found that the less
hemolytic peptides incorporated a Lys residue either at
position 1 or 10. N-terminal derivatization increased the
hemolytic activity and peptides containing a Trp were the
most hemolytic. In fact, Trp has been reported to assume a
defined orientation when binding to cholesterol leading to an
increase of hemolysis [8]. Among the most active peptides,
BP31, BP52, BP78, BP102, BP103 and BP121 followed these
structural trends and displayed a high hemolysis. Peptides
with an optimal balance between antibacterial and hemolytic
activities were BP66, BP77, BP81, BP100, BP105, BP125, and
BP126.
Antibacterial compounds can be bacteriostatic or bacter-
icial. In bacteriostatic compounds, the inhibition effect on
the target bacteria is only exerted when the compound is
present and growth is restored upon dilution or inactivation.
On the contrary, bactericidal compounds kill the cells and,
therefore, the inhibitory effect persists after releasing the
treatment. Selected peptides of the present work showed a
potent bactericidal effect against E. amylovora with D values at
concentrations around the MIC of less than an hour. These
values are similar to previous reports involving peptide
sequences of 12–20 amino acids tested against Escherichia coli,
Bacillus subtilis and Pseudomonas aeruginosa [39,45].
Protease digestion stability is a desired property in
antimicrobial peptides to assure a reasonable half-life of
the molecules in the plant environment. Proteases from
epiphytic microorganisms or intrinsic to the plant in internal
tissues may degrade antimicrobial peptides [4,16]. Peptides
with an optimal biological activity profile, BP81, BP100, BP125
and BP126, also showed good stability towards protease
degradation.

We used flowers as plant model systems for E. amylovora
infection inhibition, because they are the main entrance sites
on susceptible plants for fire blight infections [48,51]. Assays
based on detached flowers have been used in studies of
pathogen aggressiveness [15] and disease control using biocontrol agents [10,41]. The treatment of pear and apple
flowers with a set of peptides from the library that showed
optimal biological properties and good stability to proteases
significantly reduced or prevented infections of E. amylovora,
without phytotoxicity. Peptide BP100 resulted to be more
potent than the parent peptide BP76 with an effective
concentration comparable to the dose of streptomycin (50–
200 μM) currently used in field sprays for control of fire blight
and other bacterial diseases of plants [30,52]. Unlike other
reports [2], in our experiments bacteria were not mixed with
the peptides before inoculation on plant material, because this
procedure dramatically decreases the number of alive cells
that are inoculated and therefore it gives disease overcontrol.
Peptides were applied into flower hypanthia, absorbed by
plant tissues and, subsequently, the pathogen was inoculated
into the treated site. This procedure mimics preventive
treatments used for field control of fire blight, although
pathogen and environment are maintained at optimal condi-
tions for infection.

In conclusion, we have identified a set of linear undeca-
peptides highly active against E. amylovora, X. axonopodis pv.
vescicatoria, and P. syringae pv. syringae, and exhibiting low
hemolytic activity. Several analogues displayed a bactericidal
effect, low susceptibility to protease degradation and were
highly active to prevent infections of E. amylovora in flowers.
The best peptide was only slightly less effective in flowers than
streptomycin, which is the most active compound used in fire
blight control. Therefore, the application of these peptides
during the bloom period of trees may offer a good perspective
in the future management of this disease.

Acknowledgments
Rafael Ferre is the recipient of a predoctoral fellowship from
the Ministry of Education and Science of Spain (MEC). This
work was supported by grants from CICYT, CTQ2006-04410/
BQU and AGL2006-13564/AGR of MEC of Spain and CIRIT from
the Catalan Government.

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