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Bioprospection of Cationic and Anionic Antimicrobial Peptides from Bell Pepper Leaves for Inhibition of *Ralstonia solanacearum* and *Clavibacter michiganensis* ssp. *michiganensis* Growth

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Abstract

Bioprospection of natural compounds to be used as plant protection offers exciting possibilities in agribusiness. 'Samara' (SM) and 'Sunshine' (SH) cultivars of bell pepper are resistant against some plant pathogens, and leaf antimicrobial peptides could account for their defence system. In the present study, 5–7 kDa peptides were partially purified from cell wall extract (CWE) of bell pepper leaves and further resolved by ion-exchange chromatography. The cationic and anionic peptide-enriched fractions from both SM-CWE and SH-CWE strongly inhibited *in vitro* growth of *Ralstonia solanacearum* and *Clavibacter michiganensis* ssp. *michiganensis*.

Introduction

Natural compounds seem to be a rich source of defence agents against plant pathogens (Alan et al., 2004). Many of them present low toxicity to humans and animals, low environmental impact, low residues in food and compatibility with integrated pest management programmes (Knight et al., 1997; Castro and Fontes, 2005). Antimicrobial peptides are important components of the innate defence of insects, amphibians, plants and mammals (Hancock and Lehrer, 1998), and production of these small molecules corresponds to an ancient, swift, flexible, economic and widespread defence strategy (Zasloff, 2002). Biotechnologically, peptides produced by higher eukaryotes have offered exciting pharmaceutical possibilities as topical antimicrobial agents for animals and humans, as they differ structurally from conventional antibiotics produced by micro-organisms (Ganz and Lehrer, 1999). Ten groups of defence peptides have been identified in plants (Gar-

cia-Olmedo et al., 2001). In agribusiness, researches with eukaryotic peptides are intended to develop plant protection compounds (Castro and Fontes, 2005), as they generally exhibit a broad range of activity against bacteria, fungi, viruses and/or protozoa, with disruption of the membrane integrity, at low concentration (Hancock and Lehrer, 1998). Although plant diseases caused by *Ralstonia solanacearum* and *Clavibacter michiganensis* ssp. *michiganensis* represent important agricultural constraints in tropical and subtropical regions, for some solanaceous species, such as bell pepper, they do not represent serious agricultural concern (Kimati et al., 1997). This study aimed to bioprospect antimicrobial activity in peptide-enriched fractions of extracts from bell pepper leaves to be used against plant pathogens.

Materials and Methods

Plant and micro-organisms – origin, cultivation and maintenance
Seeds from two bell pepper (*Capsicum annuum*) cultivars ('Samara' – SM and 'Sunshine' – SH), obtained from the Plant Science Department, UFV, Brazil, were cultivated in the field (60 days). Fully expanded leaves were used as peptide source. The bacteria *R. solanacearum* and *C. michiganensis* ssp. *michiganensis*, obtained from the Plant Pathology Department, UFV, were maintained under mineral oil (Smith and Onions, 1994), and grown on solid LB nutrient medium (Gerhardt, 1994) at 28°C.

Peptide extraction and purification

Soluble extract (SE) and cell wall extract (CWE) were obtained from bell pepper leaves of SM or SH cultivars (Segura et al., 1998). Frozen leaves (3 g) were powdered and extracted with 12 ml of 100 mM Tris-HCl

pH 7.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine and 2 mM thiourea. After centrifugation (20 100 × g, 30 min, 4°C), the supernatant was saved, the precipitate was washed twice with water, and the resulting supernatants combined with the saved one, hereafter named SE. The precipitate was resuspended with 7.5 ml of 1.5 M LiCl, 10 mM EDTA, 1 mM PMSF, 1 mM benzamidine and 2 mM thiourea, the mixture stirred (2 h, 4°C), centrifuged and the supernatant was referred to as CWE. Every prepared extract (SM-SE, SM-CWE, SH-SE and SH-CWE) was fractionated with 35% saturated ammonium sulfate (4°C, 2 h) and the resulting supernatant was heated (80°C, 15 min) and centrifuged. Supernatants were dialysed (1000 Da; Sigma, St Louis, MO, USA) against 5 mM Tris-HCl pH 7.0, lyophilized, resuspended with 7 ml of water and fractionated by ion-exchange chromatography (IEC; fast protein liquid chromatography (FPLC); Bio-Rad, Hercules, CA, USA) on a 15 ml DEAE-Sepharose (Amersham/GE, Piscataway, NJ, USA) column equilibrated with 25 mM Tris-HCl pH 7.0, at 1.0 ml/min, in absence or presence of 1 M NaCl in the equilibrium buffer (1 ml fractions). Peptides were monitored at A_{280} . Anionic (AF) or cationic (CF) peptide-enriched fractions were pooled, dialysed and assayed for antimicrobial activity.

Peptide electrophoresis in presence of SDS and Tricine

The peptide purification level in the four prepared extracts, after salt and heating fractionations, was checked by sodium dodecyl sulfate (SDS)-Tricine-PAGE (Judd, 1994). The samples (5, 10 or 15 μ l) were boiled (4 min) in a loading buffer and carried out in three-phase gels consisting of a separating gel (16.5% T and 3.0% C), a spacer gel (9.9% T and 3.0% C) and a stacking gel (5.12% T and 2.6% C), at 100 (30 min), 80 (20 min) and 60 V (150 min). The cathode-running buffer was 0.1 M Trizma base, 0.1 M Tricine and 0.1% SDS, pH 8.25, and the anode counterpart consisted of 0.2 M Trizma base, pH 8.9. The wide range molecular weight markers (Bio-Rad) was used and the peptide bands were stained with Coomassie Brilliant Blue G250.

Antimicrobial assays

The inhibitory activity of peptide-containing fractions was evaluated against the plant pathogens *R. solanacearum* (Gram-negative) and *C. michiganensis* ssp. *michiganensis* (Gram-positive) by using the Microplate Test (Broekaert et al., 1990). Bacterial cell suspension ($A_{560} = 1.2$) from liquid LB culture medium was grown at 28°C, and 15 μ l was transferred to disposable sterile polystyrene plates (96-well plate, high binding, covered and flat bottom) containing 145 μ l of liquid LB medium (double strength) and 130 μ l of CF or AF from SM-CWE or SH-CWE. The plates were incubated at 28°C and growth was monitored at A_{560} . Only sterile culture medium and bacterial liquid cultures were used as controls. Tests were car-

ried out in three replicates. For plot presentation, initial culture A_{560} values were standardized in 0 for all assays.

Results

Bell pepper fully expanded leaves as antimicrobial peptide source

The electrophoresis fractionation of SM-CWE and SH-CWE, after salt and heating fractionations, showed the presence of intense peptide bands (around 6500 Da; Fig. 1), whereas the SM-SE and SH-SE were resolved into several high molecular weight (M_r) polypeptides (data not shown). These results demonstrated that salt and heating fractionations enriched the peptide content in CWE from both cultivars and, therefore, they were chosen to undergo IEC (Fig. 2) and antimicrobial tests (Fig. 3). After ionic fractionation (Fig. 2), one anionic (AF) and one cationic (CF) fractions were obtained for each bell pepper variety. The AF-SM presented a high protein concentration when compared with AF-SH. In contrast, the protein concentration of CF-SH was greater than CF-SM. All the four fractions were used for antimicrobial analysis.

Antimicrobial activity against plant pathogenic bacteria

Antimicrobial tests using the partially purified cationic (CF) and anionic (AF) CWE fractions from both cultivars (SM and SH) were developed against two plant pathogenic bacteria. All tested fractions (SM-CF, SM-AF, SH-CF and SH-AF) effectively inhibited growth of *R. solanacearum* (Fig. 3a) and *C. michiganensis* ssp. *michiganensis* (Fig. 3b). For CWE fractions, both SM-AF and SH-CF exhibited a total inhibition rate of *R. solanacearum* growth, while SM-CF inhibited bacterial growth at 96% and SH-AF at 81% when compared with control (without peptide; Fig. 3a). The inhibition rate of *C. michiganensis* ssp. *michiganensis*

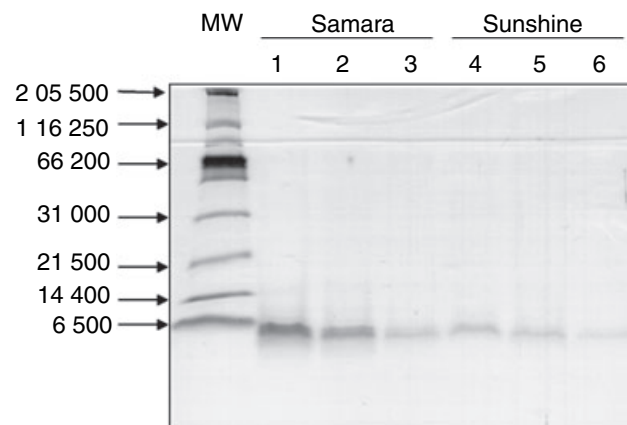


Fig. 1 Three-phase sodium dodecyl sulfate (SDS)-Tricine-PAGE of cell wall extracts (CWE) from leaves of 'Sunshine' (SH) and 'Samara' (SM) bell pepper varieties, after ammonium sulfate and heating fractionations. Samples 15, 10 and 5 μ l of SM-CWE (lines 1–3, respectively), and 15, 10 and 5 μ l of SH-CWE (lines 4–6, respectively). MW was 5 μ l of the wide range molecular weight markers (Bio-Rad, Hercules, CA, USA). Protein bands were stained with Coomassie Brilliant Blue

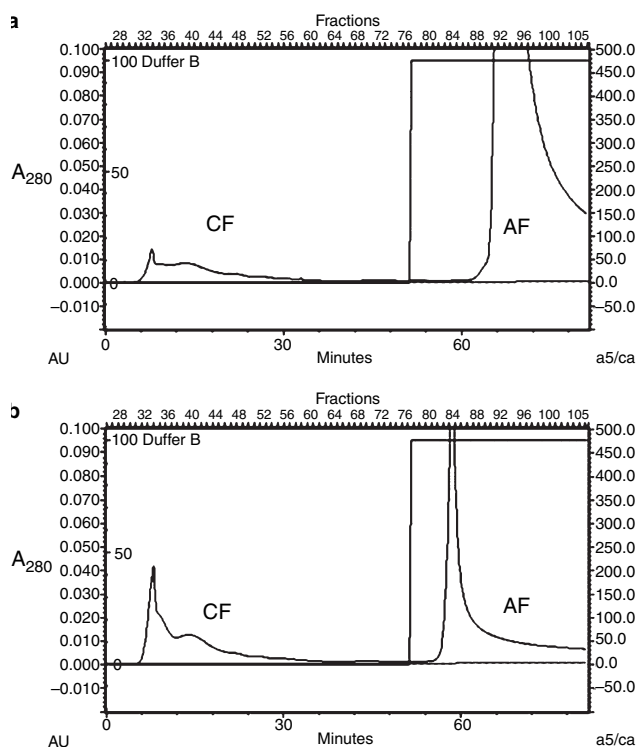


Fig. 2 Ion-exchange chromatography of cell wall extracts (CWE) from leaves of 'Samara' (a) and 'Sunshine' (b) bell pepper varieties after ammonium sulfate and heating fractionations. Samples were carried out in a DEAE-Sepharose column (15 ml), using 25 mM Tris-HCl pH 7.0, and flow rate of 1.0 ml/min, in absence (up to fraction 75) or presence (after fraction 76) of 1 M NaCl. About 1 ml fraction was collected and peptides automatically monitored at A₂₈₀. CF and AF correspond to the obtained cationic and anionic fractions respectively

growth was total for SM-AF and for SH-CF, 87% for SM-CF and 61% for SH-AF when compared with control (Fig. 3b).

Discussion

Evaluation of the antimicrobial potential of peptide-enriched fractions against two economically relevant plant pathogenic bacteria (Fig. 3) indicated that bell pepper leaves represent a good source of cationic and anionic peptides to potentially develop plant protection agents. Antimicrobial peptides are expressed in several organs of plants (Garcia-Olmedo et al., 2001); however, they have been barely detected in leaves under normal conditions. In contrast, we demonstrated that fully expanded bell pepper leaves represent a good antimicrobial peptide source, as a constitutive, pre-existing innate defence (Garcia-Olmedo et al., 2001). This defence mechanism represents advantage because these small molecules can be efficiently synthesized in a quick, economical and flexible way (Zasloff, 2002).

It should also be considered that CWEs, in general, present smaller total protein concentrations than SEs. CWE produced more homogenous peptide fractions and, thus, was selected to proceed further with new purification protocols (Fig. 2) and antimicrobial assays (Fig. 3), in contrast to SE. The higher relative expres-

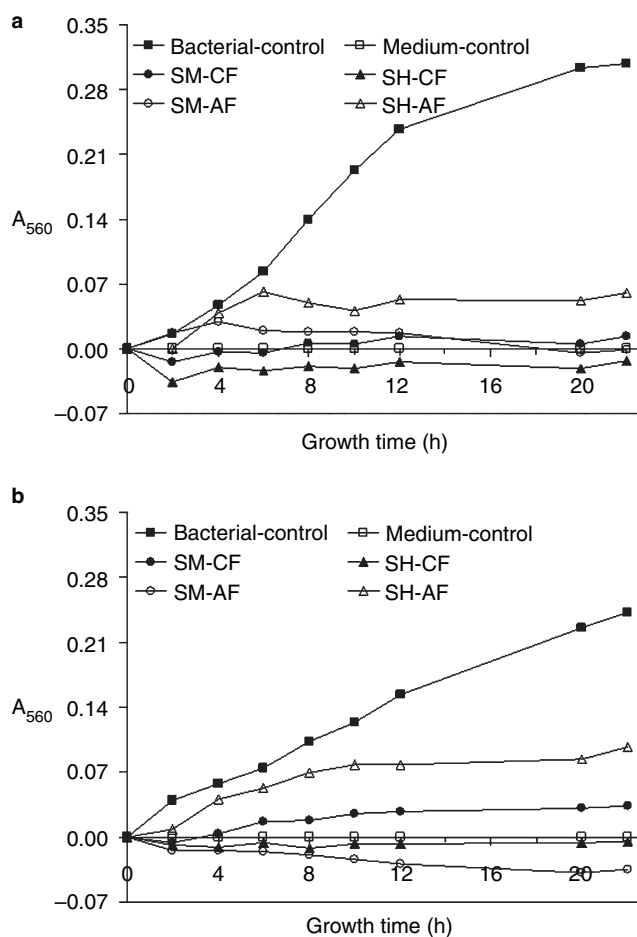


Fig. 3 Antimicrobial activity against *Ralstonia solanacearum* (a) and *Clavibacter michiganensis* ssp. *michiganensis* (b) of cationic (CF, full symbols) or anionic (AF, empty symbols) fractions of cell wall extract from leaves of 'Samara' (SM, circles) and 'Sunshine' (SH, triangles) bell pepper varieties, after ion-exchange chromatography. Bacterial control (full squares) was the bacterial liquid culture, and medium control (empty squares) was the sterile culture medium only. The plates were incubated at 28°C and the growth was monitored at A₅₆₀

sion of peptides in CWE than in SE (data not shown) is consistent with a primary defence function of the vulnerable peripheral tissues (Broekaert et al., 1995). Our results also suggest a direct correlation between the amount of peptide in the extracts (Fig. 2) and the antimicrobial activity (Fig. 3).

The high antimicrobial activity detected in AF (Figs 2 and 3) represents an interesting observation, as cationic peptides are frequently involved in the innate defence of the plants. Anionic peptides have been rarely described, especially as constitutively expressed in amphibious, with M_r lower than 1 kDa (Lai et al., 2002). Our results also underline the potential commercial application of bell pepper peptides as plant defence agents against two bacterial groups that differ in the cell coat composition, because antimicrobial peptides probably develop their activity in the microbial cell surface. These compounds could represent a new strategy in biotechnology development, when peptides might be used as natural molecules able to neut-

ralize, and/or inhibit plant pathogens without inducing resistance (Heinemann et al., 2000). Synthetic peptides could be produced or natural defensives could still be formulated by using the crude-enriched peptide extracts for application in agribusiness.

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