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Characterization of a *Capsicum chinense* Seed Peptide Fraction with Broad Antibacterial Activity

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Abstract: Habanero chili pepper (*Capsicum chinense*) is widely consumed as a fresh vegetable, although its extremely high capsaicin content has led to other uses (e.g., medicine and self-defense). Recently described antimicrobial peptides from *C. annuum* were very efficient in inhibiting growth in human and plant pathogenic bacteria and fungi. In order to explore the potential use of *Capsicum chinense* seeds as a source of antimicrobial peptides, in the present study a peptide fraction from *C. chinense* pepper seeds, denominated G10P1, was enriched, partially purified and its antimicrobial activity tested against the plant and human pathogens *Xanthomonas campestris*, *Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Erwinia carotovora*, *Agrobacterium* sp., *Shigella flexnerii*, *Escherichia coli*, *Staphyllococcus aureus* and *Bacillus subtilis*. The minimum inhibitory concentration of the G10P1 peptide fraction against *X. campestris* was 12.5 μg mL^{-1}. Electrophoresis of the G10P1 in a denaturant 15% polyacrylamide gel showed it to be composed of ~7.57 and ~5.6 kDa polypeptides, both associated with an area of strong antibacterial activity. The sequencing of 18 amino acids from the N-terminal of the ~7.57 peptides and 12 from the ~5.6 kDa peptides showed no clear association with previously described antimicrobial peptides. However, the ~5.6 kDa peptides were related to the NAC and WRKY transcription factors, both involved in direct regulation of the plant defense response against pathogen attack and the ~7.57 kDa peptides had low homology with a 3-oxo-[acyl-carrier-protein] synthase from *Capsicum chinense*.

Key words: Antimicrobial peptides, habanero pepper, seed peptides, plant antimicrobial peptides, transcription factors, column chromatography, *Capsicum chinense*

INTRODUCTION

Plants have developed a number of defense mechanisms against pathogen attack (Lay and Anderson, 2005). Among these is the innate response, an ancient mechanism that provides relatively rapid host protection with low energy and biomass expenditure. This response is often afforded by small proteins with antimicrobial properties and broad bioactivity spectrums (Hancock and Lehrer, 1998; Zasloff, 2002).

Antimicrobial peptides offer many exciting possibilities in agricultural and pharmaceutical research for development of plant protection compounds (Castro and Fontes, 2005) and new antibiotics (Mygind *et al*., 2005). This is because they generally exhibit a broad range of activity against bacteria,

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fungi, viruses and/or protozoa by disrupting membrane integrity, even at low concentrations (Hancock and Lehrer, 1998; Mygind *et al*., 2005). An additional promising quality is that antimicrobial peptides do not apparently induce resistance in the microorganisms exposed to them (Zasloff, 2002), a side effect common among pathogenic agents after frequent exposure to agrochemicals.

Control of fungi and bacteria pathogenic to plants currently relies mainly on broad-spectrum agrochemicals (Agrios, 2005; Moguel-Salazar *et al*., 2007). Unfortunately, many of these compounds are largely non-selective, toxic to humans and animals and often have negative environmental impacts (Montesinos, 2007).

In tropical and subtropical regions, bacterial pathogens such as *Ralstonia solanacearum*, *Clavibacter michiganensis* sp., michiganensis, *Xanthomonas campestris* pv *vesicatoria* and *Pseudomonas* sp., pose serious pathogenic threats to species such as tomato, pepper and zucchini (Kimati *et al*., 1997; Moguel-Salazar *et al*., 2007). Plant antimicrobial peptides represent a promising alternative to agrochemicals for control of plant pathogens like those above. However, it is first necessary to identify sources which contain these peptides in large quantities. *Capsicum* species (i.e., peppers) are commonly grown in warm, humid regions such as the tropics and subtropics and their fruit are mainly used in local cuisine. Recent reports state that the *Capsicum* genus, among other genera, is an apparently good source of antimicrobial and antifungal peptides, since these compounds have been isolated from the cell walls of pepper leaves (Texeira *et al*., 2006) and seeds (Diz *et al*., 2006). In these studies, 5-7 kDa cationic and anionic peptides were isolated from pepper leaves and a 9.46 kDa Lipid Transfer Protein (LTP) was isolated from pepper seeds. The cationic and anionic peptides strongly inhibited *in vitro* growth of *Ralstonia solanacearum* and *Clavibacter michiganensis* (Texeira *et al*., 2006), while the LTP inhibited yeast (*Sacharomycces cerevisiae*) growth by yeast plasma membrane permeabilization (Diz *et al*., 2006). Additionally, analysis of another chili pepper antimicrobial peptide (6-8 kDa) indicated it did not match any previously described antimicrobial protein, suggesting that chili peppers may contain antimicrobial peptides that have yet to be described.

No research exists on the existence of antimicrobial peptides in the tissue of habanero chili pepper (*Capsicum chinense* Jacq), although a gene encoding for a defensin or gamma-thionin has been cloned and described (Aluru *et al*., 1999). It is also known, however, that the ancient Mayans used different parts of the habanero chili pepper plant to combat infections of the skin, teeth, stomach wounds and fresh burns (Cichewicz and Thorpe, 1996), suggesting the presence of some kind of antimicrobial compound. To date, the antimicrobial effect of habanero chili is mainly ascribed to its capsaicin and dihydrocapsaicin content, both of which have a high degree of biological activity (Surh and Lee, 1995; Islas-Flores *et al*., 2005).

Capsicum chinense fruit is used in a limited number of applications, mainly processing for its oleoresins and capsaicin and as a fresh vegetable in the food industry. As part of an ongoing effort to diversify the uses of *C. chinense*, we have been studying its potential as an antimicrobial peptide source, which could expand its use as an ingredient in natural agrochemical and/or medical applications. In the present study we report on the biological activity of an enriched low molecular weight peptide fraction extracted from *C. chinense* seeds which is composed of ~7.57 and ~5.6 kDa peptides. Denominated G10P1, it strongly inhibited the growth of plant and human bacterial pathogens in *in vitro* assays.

MATERIALS AND METHODS

This study was carried out from January, 2006 to January, 2008, in the laboratory No. 06, of the Unidad de Bioquímica y Biología Molecular de Plantas from Centro de Investigación Científica de Yucatán, A.C., placed in Mérida, Yucatán, México.

Protein Extraction

Capsicum chinense seeds were removed from green ripe fresh fruits (2 kg) purchased in a local market. Forty grams of *C. chinense* seeds with liquid nitrogen were ground in a coffee mill until a fine powder was produced. This powder was homogenized with 7.5 mL buffer A (50 mM Tris-HCl at pH 7.5, added to 1 mM NaCl, plus one tablet protease inhibitor cocktail (Complete Inhibitor, Roche), the homogenate centrifuged at 40,000 x g for 20 min and the supernatant recovered. The sediment (cellular debris) was resuspended and reextracted with 7.5 mL buffer A, centrifuged again as above, the supernatant recovered and the sediment discarded. The two supernatants (~10 mL) were mixed and centrifuged at 100,000 x g for 45 min, the resulting supernatant (the soluble fraction) recovered and stored at -80°C until use.

Protein Purification

The soluble fraction (1.2 mL) was loaded into a 1.0×100 cm chromatography column (Glass Econo-column, Biorad) containing a bed of 40 mL of Sephadex-G50 resin (Pharmacia) earlierly equilibrated with buffer A. The sample was eluted at 15 mL h^{-1} and each 0.75 mL fraction collected in an automata sized fraction collector (Pharmacia). Eluted fractions were tested for the presence of proteins at 280 nm in a Genesis 10 uv spectrophotometer (ThermoSpectronic). Protein-containing fractions occurred in two sets of tubes (i.e., 17 to 30 and 32 to 68). The fractions in each set were mixed and denominated as peaks G50P1 and G50P2, respectively. Protein samples taken from both peaks were tested for their inhibitory effect on growth in *Xanthomonas campestris* (ATCC 10544). Peak G50P2 exhibited the strongest inhibitory effect and so it was lyophilized and resuspended in 0.25 mL buffer A. This chromatographic protocol was repeated several times and all the resulting G50P2 resuspended samples mixed. A sample (1.0 mL) of this G50P2 solution was loaded into a second 1.0×100 cm chromatography column (Glass Econo-column, Biorad) containing a bed of 40 mL Sephadex-G10 resin (Pharmacia) previously equilibrated with buffer A. This sample was eluted as described above and the fractions tested for protein at 280 nm. The protein-containing fractions (19-30) were mixed, denominated peak G10P1 and lyophilized. This lyophilized G10P1 was resuspended in 0.25 mL buffer A, its protein content quantified and its inhibitory effect tested on growth in *X. campestris* and other bacteria.

Bacterial Growth Inhibition Assays

Nine bacteria (*Xanthomonas campestris* (ATCC, 10544), *Pseudomonas aeruginosa* (PAO1 strain), *Agrobacterium* sp., (ATCC BAA-101) *Pseudomonas syringae* (ATCC 11043), *Erwinia carotovora* (ATCC 15713), *Shigella flexnerii* (ATCC 9728), *Escherichia coli* (ATCC 128), *Staphyllococcus aureus* (ATCC 4012) and *Bacillus subtilis* (ATCC 6633) were grown in solid bacterial screening medium (BSM, Sigma) for 24 h at 25°C. A sample of each was transferred to 1 mL isotonic solution (146 mM NaCl), the bacteria population adjusted to 1×10^8 UFC (0.1 absorbance units at 600 nm) and this volume mixed with warm (37°C), liquid BSM culture medium. Two petri dishes per bacterial strain were loaded with 20 mL bacteria-inoculated BSM culture medium and placed in a sterile laminar flow cabinet for 20 min, until the culture medium solidified. Four sterile Whatman paper filter disks (0.5 cm diameter×2 mm wide) were placed in each petri dish: one loaded with ampicillin (40 µg 0.1 mL^{-1}) and the remaining three loaded with a *C. chinense* peptide G10P1 fraction (25, 30 or 50 µg 0.1 mL^{-1}). After 10 min the petri dishes were covered and left to incubate for 24 h at 25°C. The inhibitory effect of each substance and/or concentration was then evaluated by visually measuring the inhibition areas with a Vernier rule.

Minimum Inhibitory Peptide Concentration

Using *Xanthomonas campestris*, bacterial screening medium was inoculated with 1×10^8 UFC and plated in petri dishes as described above for the bacterial growth inhibition assays. In the minimum

inhibitory concentration assay, however, increasing concentrations (from 2 to 50 μg) of enriched G10P1 peptide fraction (100 μL final volume) were added to sterile filter paper placed on the solidified BSM medium. After 24 h, the inhibition zone diameter of each peptide concentration was measured with a Vernier rule.

Protein Determination

A Bradford assay (Bradford, 1976) with bovine serum albumin fraction V (Sigma) as a standard was used to quantify protein content.

Inhibition of Growth in *Xanthomonas campestris* after Protein Electrophoresis in Denaturant Polyacrylamide Gel Electrophoresis

A protein sample (50 μg) from the G10P1 fraction was added to enough volume of Laemmli buffer (Laemmli, 1970) to complete a 1X concentration of the buffer. This was heated to 95°C for 5 min and placed on ice. The protein sample was then separated by electrophoresis in 15% SDS-PAGE at 100 V for 3 h. After electrophoresis, the gel was placed in a sterile airflow cabinet and washed every two hours in sterile distilled water (four water changes total). The gel was placed in a sterile plastic petri dish, loaded with liquid BSM medium inoculated with *X. campestris* (1×10^8 UFC) and the medium allowed to solidify. Three sterile filter paper disks were then placed next to the polyacrylamide gel and individually loaded with 0.1 mL buffer A, 0.1 mL (40 μg) ampicillin or 0.1 mL (50 μg) G10P1 fraction (one per disk). The dish was covered, incubated at 25°C for 24 h and inhibition of *X. campestris* growth measured.

Amino Acid Terminal Sequencing

Partially purified G10P1 was loaded in 15% SDS-PAGE and electrophoresed at 100 V for 4 h. The separated protein pattern was electroblotted from the polyacrylamide gel to PVDF-membrane using a buffer (0.024 M Trizma-base; 0.1875 M Glycine; 20 % methanol) at 50 V for 12 h (overnight). The membranes were stained with Ponceau's red, the areas at ~7.57 and ~5.6 kDa removed and these subjected individually to an. automatic Edman degradation in a Beckman LF 3000 Protein Sequencer (Palo Alto, CA, USA).

RESULTS

Protein Purification

Fractionation of the protein contained in the soluble fraction using the Sephadex G-50 column showed two peaks based on the fraction number at which they were eluted: G50P1 (Fig. 1a, number 1) and G50P2 (Fig. 1a, number 2). Fraction G50P1 eluted in a small number of tubes and formed a sharp peak, while G50P2 eluted in a larger number of tubes and formed a broad peak. No protein was present in the collected fraction after tube 70. Analysis in 15% SDS-PAGE of the protein contained in the G50P1 and G50P2 fractions showed G50P1 to be not very complex (Fig. 1c, lane 1), although it contained more polypeptides than G50P2 (Fig. 1c, lane 2).

The G50P2 fraction exhibited the highest inhibitory effect on *Xanthomonas campestris* (ATCC 10544), so protein (367.65 μg 1.25 mL^{-1}) from this peak was fractionated in a Sephadex G-10 column. After elution, the G50P2 decomposed into two new peaks, based on their elution: G10P1 (Fig. 1b, lane 3) and G10P2 (Fig. 1b, lane 4). The G10P1 fraction had a sharp peak and contained the ~7.57 and ~5.6 kDa peptides shown to be in this sample by the 15% SDS-PAGE (Fig. 1b, lane 3). In contrast, the G10P2 eluted as a broad peak and contained none of the proteins identified in the 15% SDS-PAGE (Fig. 1c, lane 4).

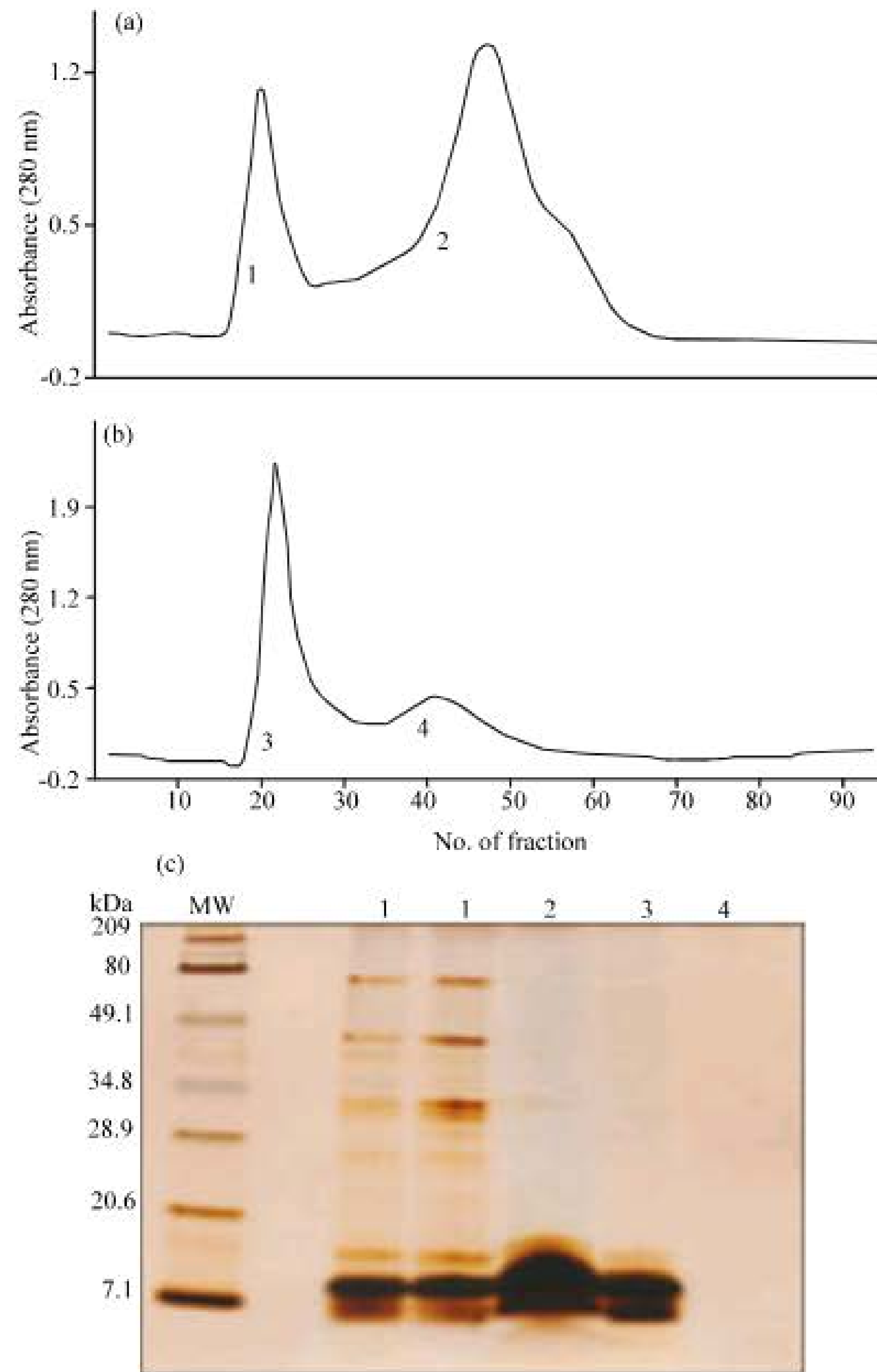

Fig. 1: Soluble protein (360 μg) was fractionated in Sephadex (a) G-50 or (b) G-10 columns. (c) Protein samples (3 μg) taken from each mixed fraction were electrophoresed in 15% SDS-PAGE and stained with silver. MW: Molecular weight marker, 1: G50P1, 2: G50P2, 3: G10P1, 4: G10P2

The minimum protein concentration of the G10P1 fraction that effectively inhibited growth in *X. campestris* was determined by testing the effect of different protein concentrations in a 100 μL final volume. All peptide-containing samples inhibited growth in *X. campestris*. Inhibition area diameter varied depending on peptide concentration, with the smallest diameters at concentrations below 10 μg, increased inhibition diameters at levels above 12.5 μg and the largest diameters (and highest inhibition) at concentrations greater than 20 μg (Fig. 2).

Once minimum inhibitory concentration was determined, 50 μg of the G10P1 fraction were fractionated by electrophoresis in 15% SDS-PAGE. The resulting gel was placed in a petri dish, the dish loaded with liquid BSM medium inoculated with *X. campestris* (1×10^8 UFC) and incubated for

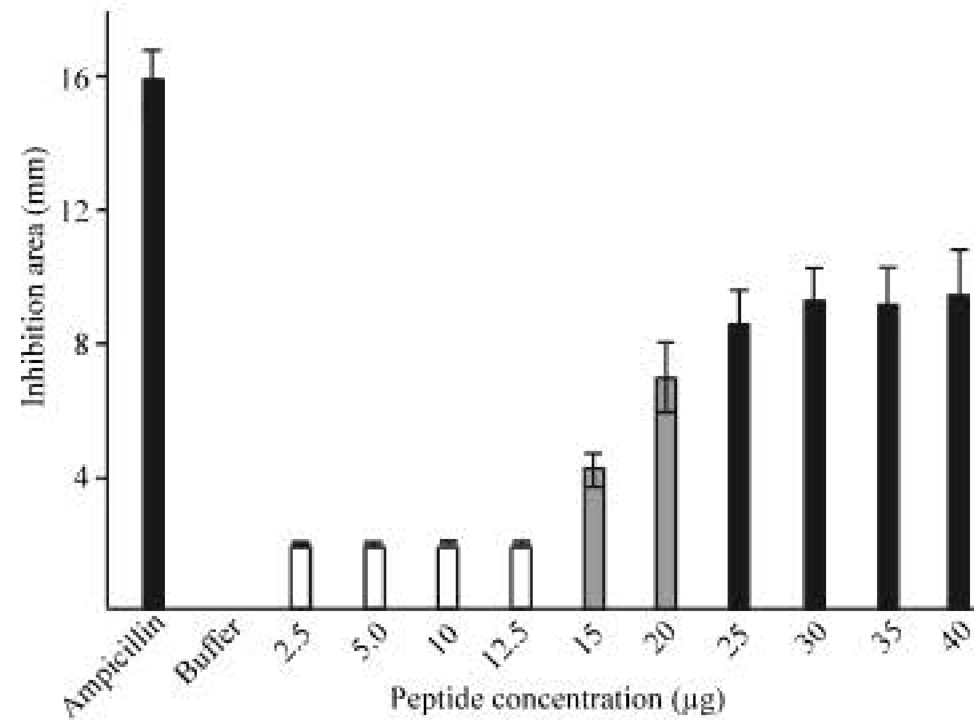

Fig. 2: Minimum inhibitory concentration of G10P1 that inhibits growth in *Xanthomonas campestris* (ATCC 10547). Increasing concentrations (2.5-50 μg) of the G10P1 fraction were loaded into filter disks and the inhibitory effect on bacterial growth determined after 24 h. The positive control was 40 μg ampicillin and the negative control 100 μL extraction buffer containing no protein. Bar tone indicates inhibition level (darker = higher)

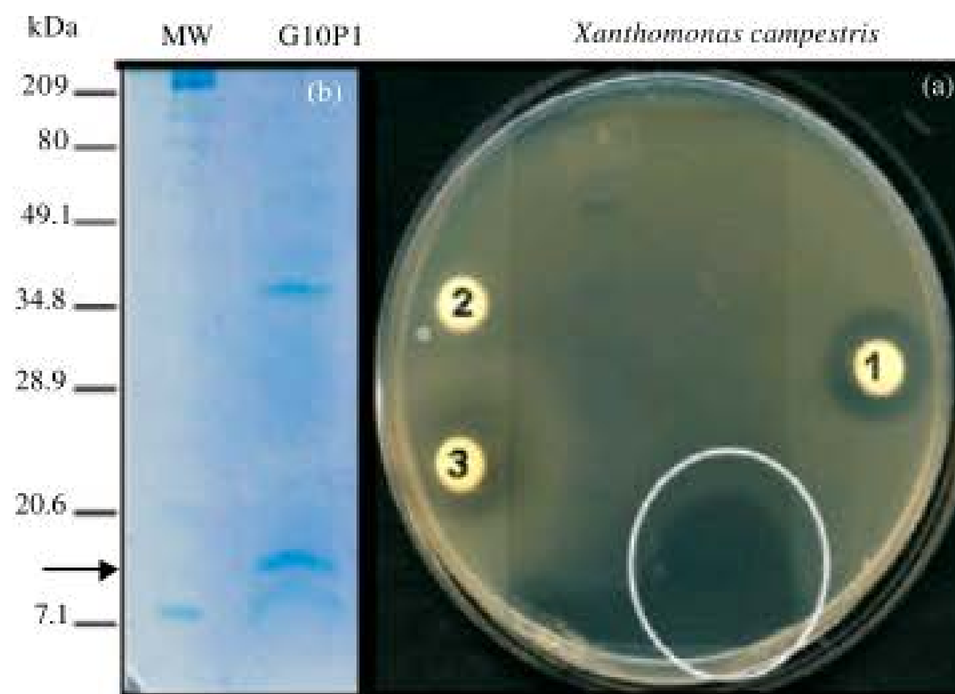

Fig. 3: Stability of G10P1 fraction inhibitory effect on growth in *Xanthomonas campestris* (ATCC 10547). Protein (50 μg) from the G10P1 fraction was electrophoresed in 15% SDS-PAGE and the resulting gel washed. (a) It was then placed in a Petri dish and covered with bacterial screening media containing 1×10^8 UFC *Xanthomonas campestris*. Bacterial growth was evaluated after 24 h. A parallel gel containing an identical protein sample (50 μg) from G10P1 was stained with Coomassie blue (b). 1 = 40 μg ampicillin; 2 = 0.1 mL extraction buffer; 3 = 50 μg 0.1 mL^{-1} G10P1 fraction; black arrow indicates position of electrophoretical migration of polypeptides with antimicrobial activity; white circle = area where ~7.57 and ~5.6 kDa polypeptides migrated, with strong inhibition

24 h. The area including migration of ~7.57 and ~5.6 kDa polypeptides (Fig 3a, arrow) exhibited strong inhibition (Fig. 3b, circled area). Similar growth inhibition patterns in *X. campestris* were

observed on paper disks containing 40 µg 0.1 mL^{-1} ampicillin (Fig. 3b, number 1) or 50 µg G10P1 peptide sample (Fig. 3b, number 3), but no inhibitory effect was observed on disks containing only buffer A (Fig. 3b, number 2).

Growth Inhibition in Different Human and Plant Bacterial Pathogens

The range of bacterial microorganisms over which the G10P1 *C. chinense* fraction exhibited an inhibitory effect was tested using 9 bacterial strains (both gram positive and negative). The *Capsicum chinense* G10P1 fraction was applied at 25, 30 and 50 µg 0.1 mL^{-1} concentrations, 0.1 mL buffer A was used as a negative control and 40 µg 0.1 mL^{-1} ampicillin as a positive control. At 30 and 50 µg, the G10P1 fraction exerted a greater inhibitory effect on growth in *Pseudomonas aeruginosa* (PAO1 strain), *Erwinia carotovora* and *Agrobacterium* sp., than did ampicillin at the tested concentration (Table 1). In contrast, ampicillin induced the highest growth inhibition levels in the remaining bacteria (*Pseudomonas syringae*, *Xanthomonas campestris*, *Shigella flexnerii*, *Escherichia coli*, *Bacillus subtilis* and *Staphyllococcus aureus*) (Table 1).

Amino Acid Terminal Sequencing

Sequencing of the amino terminals of the ~7.57 and ~5.6 kDa peptides in the G10P1 fraction by Edman-reaction after separation by electrophoresis in 15% SDS-PAGE and electroblotting onto PVDF membrane (Fig. 4), showed the ~7.57 and ~5.6 kDa peptides to have an N-terminal amino acid sequence: AVCAQTDAQVDADLAPQV and AQCSAQVLSPXN, respectively. A BLAST analysis comparing both N-terminal amino sequences to protein amino acid sequences on deposit in the National Center for Biotechnology Information (NCBI) database showed that neither *C. chinense* peptide exhibited strong homology with any previously reported plant antimicrobial peptides. However, the ~5.6 kDa peptide did have homology with a NAC transcription factor from *Hordeum vulgare* ssp *vulgare* (Table 2), which is a positive regulator of penetration resistance in barley and *Arabidopsis* (Jensen *et al*., 2007) and with a WRKY transcription factor from *Arabidopsis* (Zheng *et al*., 2007). The ~7.57 kDa peptide showed low homology with a 3-oxo-[acyl-carrier-protein] synthase from *Capsicum chinense* (Table 2) (Aluru *et al*., 1998).

Table 1: Inhibitory growth effect of the G10P1 fraction against different pathogenic microorganisms. Increasing concentrations of G10P1 were added to the initial inoculums (1×10^{8} UFC) of individual microorganisms, allowed to incubate for 24 h and the inhibition areas recorded. Ampicillin and extraction buffer were used as positive and negative controls, respectively

	Inhibition area (mm)			
Microorganism	Ampicillin (40 µg mL^{-1})	Peptide (25 µg mL^{-1})	Peptide (30 µg mL^{-1})	Peptide (50 µg mL^{-1})
Pseudomonas aeruginosa	7.5±0.05	6.60±2.60	10.00±3.00	13.0±1.78
Pseudomonas syringae	20.6±2.90	14.00±4.90	13.60±3.50	18.5±3.70
Xanthomonas campestris	13.5±1.80	6.16±1.16	7.50±1.87	8.8±1.60
Erwinia carotovora	8.8±0.40	7.00±0.66	9.16±1.72	10.3±2.80
Agrobacterium sp.	5.8±0.70	5.80±0.60	7.00±0.60	10.1±2.10
Shigella flexnerii	17.0±1.67	7.16±0.75	9.16±0.75	10.1±1.16
Escherichia coli	15.1±1.16	6.50±0.80	8.30±1.50	10.1±1.16
Bacillus subtilis	22.3±0.81	8.30±1.03	10.00±1.09	11.6±2.40
Staphyllococcus aureus	14.5±1.04	6.50±0.54	6.50±0.83	8.0±0.89

Table 2: Comparison and alignment of amino acid sequences of the ~5.6 and ~7.57 kDa peptides in the G10P1 fraction from *Capsicum chinense* seeds with the amino acid sequences of the *Hv*NAC6 and WRKY transcription factors and 3-oxo-[acyl-carrier-protein] synthase from *C. chinense*

Species	Amino acid sequence	Accession No.
Capsicum chinense ~5.6 kDa peptide	QCSAQVLSP	
Hordeum vulgare ssp vulgare	235 CSEQVLSP 242	CAM59978.1
Capsicum annuum	65 QCSAQVLS 72	AAW67002.1
Capsicum chinense ~7.5 kDa peptide	AVCDAVDADLAPQV	
Capsicum chinense	445 DLEPQV 450	ACC78479.1

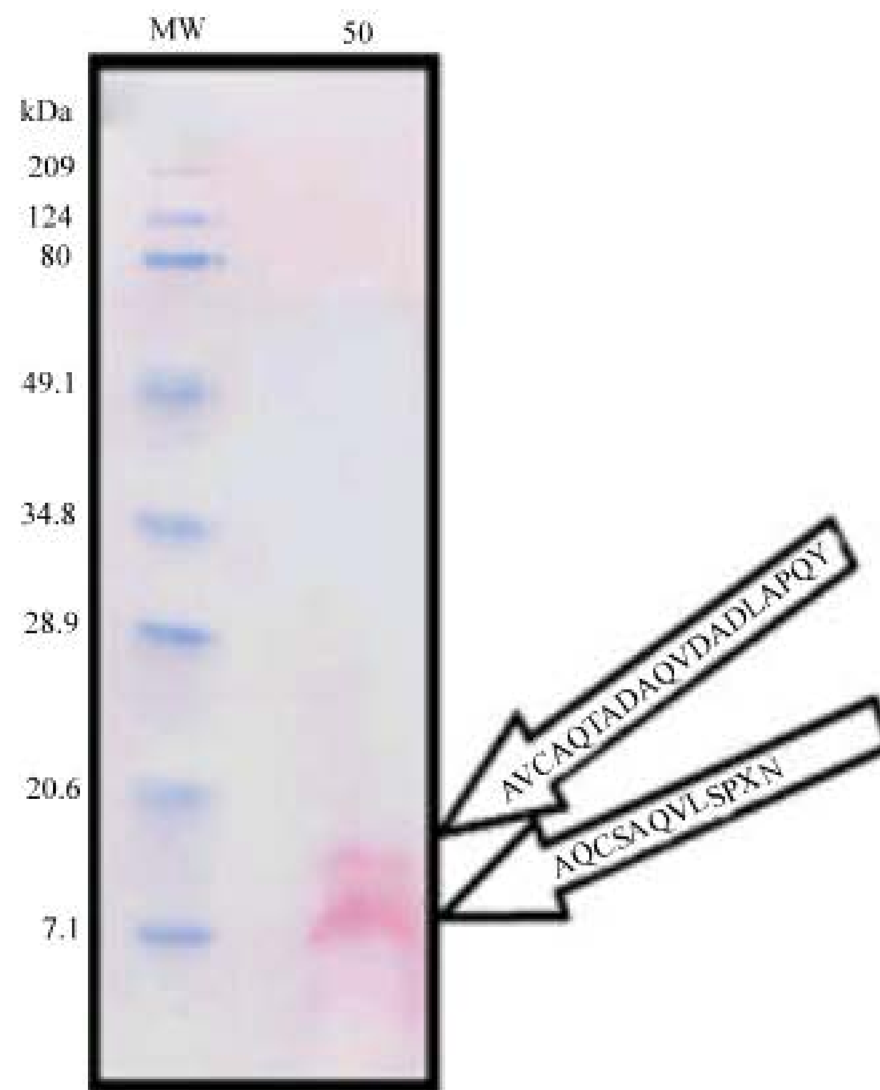

Fig. 4: The G10P1 fraction stained with Ponceau's red after electroblotting onto PVDF membrane. Protein (50 μg) in the G10P1 fraction was electrophoresed in 15% SDS-PAGE, electroblotted onto PVDF membrane and stained with Ponceau's red. MW: Wide range molecular weight protein marker, 50: G10P1 fraction; arrows indicate positions of peptide and its amino acid terminal sequence after sequencing

DISCUSSION

Chili peppers are important food ingredients, but also clearly have nutritional and medicinal properties. The ancient Maya recognized these properties and used them therapeutically. Ethnobotanical data suggest that the *Capsicum* species in particular harbor many potentially economically significant compounds yet to be discovered (Cichewicz and Thorpe, 1996). It remains unclear exactly which properties led the ancient Maya to include *Capsicum* species in their pharmacopoeia and keeps them in use by traditional cultures (Alcorn, 1984), but it may have been in response to their therapeutic properties as antimicrobial and anti-hemolytic agents. The presence of the secondary metabolite capsaicin in these species has long been associated with strong analgesic properties (Cordell and Araujo, 1993), alterations in the pH of gastrointestinal tract epithelial cells, prevention of microbial infection (Tellez *et al*., 1993) and possible anticarcinogenic effects (Surh and Lee, 1995). Recent study, however, has shown that chili species also contain peptides with strong antimicrobial activity and that these peptides are encoded in the chili genome (Texeira *et al*., 2006; Diz *et al*., 2006).

Habanero pepper (*Capsicum chinense* Jacq.) was shown here to contain two peptides with molecular masses of ~5.6 and ~7.57 kDa. These were enriched and partially purified after two simple chromatographic separation steps using Sephadex G-50 and Sephadex G-10 (Fig. 1a, b), producing a fraction we called G10P1. Similar results were reported by Diz *et al*. (2006), who purified three peptides (molecular weight 6-10 kDa) from *C. annuum* seeds using reverse phase chromatography. All

these peptides exhibited strong antimicrobial activity, but only the 10 kDa polypeptide was related to Lipid Transfer Proteins (LTPs).

In vitro growth inhibition assays with *X. campestris* (ATCC 10547) showed the minimum inhibitory concentration of the G10P1 fraction to be 12.5 μg mL^{-1} (Fig. 2), with all concentrations higher than this completely eliminating *X. campestris* from the area loaded with G10P1. This minimum inhibitory concentration is lower than that reported for peptides from *Capsicum annuum* seeds, which required 70-150 μg to inhibit growth in fungi including *Fusarium oxysporum Saccharomyces cerevisiae*, *Saccharomyces pombe* and *Candida albicans* (Diz *et al*., 2006). Unfortunately, no data was provided in this study on the antimicrobial activity of these *C. annuum* seed peptides against pathogenic bacteria.

After fractionation in 15% SDS-PAGE, the G10P1 retained its antimicrobial activity, since it still inhibited growth in *X. campestris* in the area surrounding the migration of the ~5.6 and ~7.5 kDa peptides (Fig. 3a). Similar results were obtained in assays done with finotin, an insecticidal protein isolated from the seeds of *Clitoria ternatea*, a tropical forage legume (Kelemu *et al*., 2004).

The partially purified G10P1 strongly inhibited growth in the opportunistic human pathogens *Staphyllococcus aureus*, *Escherichia coli* and *Shigella flexnerii* (Table 1), in the plant pathogens *Pseudomonas syringae*, *Xanthomonas campestris*, *Erwinia carotovora*, *Agrobacterium* sp. and *Bacillus subtilis* and in the plant/human pathogen *Pseudomonas aeruginosa* (Table 1). In *B. subtilis*, this effect was unexpected since antimicrobial peptides only act on pathogenic bacteria. The *Bacillus* species are common bacteria found in natural environments including soil, water, plant and animal tissues and *Bacillus thuringiensis* is actually used as a biological control agent, meaning it probably has little pathogenic potential (Cichewicz and Thorpe, 1996). Nonetheless, both *B. cereus* and *B. subtilis* have been known to act as primary invader or secondary infectious agents in a number of diseases and have been implicated in some cases of food poisoning (Turnbull and Kramer, 1991). This behavior in *B. subtilis* may explain why the G10P1 fraction strongly decreased growth in this gram positive bacterium.

The N terminal amino acid sequence of the ~5.6 kDa peptide (AVCAQTDAQVDADLAPQV) demonstrated that it is related to the NAC transcription factor family. It is particularly related to *Hv*NAC6, which, in *Hordeum vulgare* and *Arabidopsis*, stimulates resistance to penetration by microbial organisms through direct positive regulation of the basal plant innate response (Jensen *et al*., 2007). Although, the ~5.6 kDa peptide was apparently similar to the *Capsicum annuum* transcription factor it manifested little homology with it. Direct regulation of plant defense response against *Pseudomonas syringae* is known to involve WRKY25 in *Arabidopsis thaliana* (Zheng *et al*., 2007). In response, *P. syringae* was included in the growth inhibition assays here, in which it was effectively inhibited, perhaps due to a direct interaction between the ~5.6 kDa transcription factor peptide and bacterium constituents.

The identity of the ~7.57 peptide was not clearly defined in the present study, although 18 N-terminal amino acids were identified, which is almost 40% of its total amino acid sequence. This peptide shares some homology with a 3-oxo-[acyl-carrier-protein] synthase from *Capsicum chinense* (Aluru *et al*., 1998); therefore, it is possible it maintains some of its acyl carrier activity and thus disaggregates the bacterial membrane.

No unequivocal relationship between the peptides isolated in the present study and previously reported antimicrobial peptides could be established. This is not surprising given that the number of new described antimicrobial peptides increases almost daily (Montesinos, 2007). It is therefore possible that the ~5.6 and ~7.57 kDa peptides described here are constituents of a new class of antimicrobial peptides in the *Capsicum* genera that have not yet been described. This is supported by the results of Diz *et al*. (2006), who purified three low molecular weight antimicrobial peptides from *C. annuum* seeds, only one of which was found to be related to lipid transfer proteins. A full amino

acid sequence for the ~5.6 and ~7.57 peptides will be needed to discard or confirm this possibility and we are currently carrying out these experiments.

The present results both confirm and highlight the potential of habanero chili pepper (*Capsicum chinense*) seeds as a source of commercial antimicrobial peptides which may have pharmaceutical and agricultural applications. Furthermore, our experimental findings clearly show that *C. chinense* seeds could be used in alternative processes besides their traditional uses as germplasm.

In conclusion, the use of *C. chinense* seeds as a source of antimicrobials is supported by the positive growth inhibition of the common plant and human pathogenic bacteria by the G10P1 fraction. The peptides in the G10P1 fraction may also have potential economic advantages over commercial synthetic agrochemicals and medicines since its extraction from pepper seeds is far less expensive than the chemical synthesis usually required to manufacture artificial compounds (Zasloff, 2002). Because these peptides are small molecules-they are apparently a constitutive element of the species' preexisting innate defense system-they can be efficiently isolated using rapid, low-cost and flexible methods (Zasloff, 2002). Once isolated, these peptides, or an enriched G10P1 fraction, could then be included in biotechnology development strategies such as the formulation of natural defensive compounds (e.g., creams) for topical use in humans and/or animals, or inclusion in directly applied or dilute solutions in agriculture.

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