ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (2): 248-253, 2009
ISSN 1682-296X

Antimicrobial Peptides in Aqueous and Ethanolic Extracts from Microbial, Plant and Fermented Sources

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Abstract: The objective of this research was to isolate novel peptides from extracts prepared from native microbial, plant and fermented sources. The antimicrobial properties of these extracts were initially tested using *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus cereus*. The exact species and strains of these test microorganisms were confirmed by identifying its 16S RNA sequence. The most pronounced inhibition zone for ethanolic extracts was obtained with *Andrographis paniculata*. For peptide/protein extracts only *Allium sativum* showed promising results. The particular compound responsible for the inhibition in each case is undergoing characterization by using High Performance Liquid Chromatography (HPLC) and mass spectrometry.

Key words: Antimicrobial peptides, antibiotics, ethanolic extracts

INTRODUCTION

Biologically active peptides and polypeptides occur in a vast range of sizes and no generalization can be made about the molecular weights in relation to their functional properties. Naturally occurring peptides range in length from two amino acids to many thousands of residues. Even the smallest peptides can have biologically important effects.

A variety of peptides and proteins have been used to produce biopesticides, biopesticidal microbes and pest-resistant crops. These compounds derive from a number of sources including the venoms of predatory or parasitoid animals (Taniai, 2002), arthropod-pathogenic microbes including bacterial symbiotes of entomopathogenic nematodes (Beard, 2001), plant lectins, protease inhibitors (Brunelle *et al*., 2005) or ribosome inactivating proteins (Sharma, 2004), arthropod hormones and neuropeptides (Altstein, 2004; Borovsky, 2003), plant defensins (Lay and Anderson, 2005) and plant hormones (Dinan, 2001).

The gene-encoded cationic antimicrobial peptides (AMPs) are important mediators in the primary host defense system against pathogenic microorganisms, which are widely distributed in nature. In the last few years, the burgeoning reports of the occurrence and characterization of low-molecular-mass AMPs from a wide variety of organisms have been accumulating at a rapid rate because of their biochemical diversity, broad specificity against bacteria or fungi (Sitaram and Nagaraj, 2002) and also because some of them have anti-viral (Sitaram and Nagaraj, 2002), anti-tumoral (Rozek *et al*., 2000) or wound-healing effects (Fernandes *et al*., 2002).

On the other hand, the resistance to antibiotics of bacteria has also risen dramatically and the resistance to most or all available agents has appeared in the clinic over the past decade. There is a growing need to discover and introduce new drugs and AMPs provide new promising candidates for screening of new antibiotics.

This study was undertaken to isolate novel peptides and secondary metabolites from selected Malaysian indigenous microbial, plant and fermented sources. Subsequently, these peptides and secondary metabolites were tested *in vitro* using test microorganisms.

MATERIALS AND METHODS

This study was conducted at the Fermentation Technology Laboratory, Division of Microbiology, Institute of Biological Sciences, Faculty of Science and Department of Molecular Medicine, Faculty of Medicine, University of Malaya. The research project was conducted from September 2007 to August 2008.

Sample preparation: Plant samples were cleaned and dried at a temperature not exceeding 40°C and pulverised to powder form. Fermented samples were prepared by exposing them to solid state lactic acid fermentation at

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20% moisture. These test samples were prepared in triplicates and also used for the subsequent part of this study.

Ethanol extraction: The powder was soaked in 95% ethanol for a week. The extracts were filtered and evaporated to dryness under reduced pressure at 40°C in a rotary evaporator and then weighed to determine the total extractable compounds. The crude extracts were then transferred to vials and kept at -4°C. These crude extracts were dissolved in water or solvents and used for the assessment of antimicrobial activity (Seveno *et al*., 2008).

Peptide/protein extraction: Tissue was placed in a cold mortar and pestle. Approximately 2 mL of extraction buffer was added for every 1 g of tissue. The extraction buffer consists of 5 mL of KPO_4, 0.5 M of EDTA, 1 mL of triton X-100, 12.5 mL of 80% (v/v) glycerol and 15.4 mg of DTT for every 100 mL. The tissue was grounded till no more tissue was visible. All steps were carried out at 4°C. The ground tissues were transferred into a centrifuge tube and centrifuged at 12,000 rpm for 15 min. The pellet was discarded and the supernatant was collected and stored at -20°C. The samples were freeze dried prior and reconstituted into 5 mL of buffer before protein determination.

Protein quantification: Protein standards of appropriate concentration in the same buffer as the sample was prepared using bovine serum albumin (Sigma-Aldrich Inc., Saint Louis). The protein standards ranged from 0.1 till 1.4 mg mL^{-1}. After adding 3 mL of Bradford Reagent to each tube, these were vortexed gently for thorough mixing (Bradford, 1976). The samples were incubated at room temperature for 15 min and absorbance was measured at 595 nm. The protein concentration was determined by comparison of the measured absorbance to a standard curve prepared using the protein standards (Bradford Reagent product manual, Sigma-Aldrich, Inc., Saint Louis).

Microbial strain identification of test microorganisms: The microbial DNA was extracted using either the i-genomic BYF DNA Extraction Kit for gram positive bacteria or the i-genomic CTB DNA Extraction Kit for gram negative bacteria, (iNtRON Biotechnology, Seongnam). The extracted genomic DNA was examined by electrophoresis in a 1% agarose gel. Universal primers used to amplify 16S rRNA gene were (27f : 5'-AGA GTT TGA TCA TGG CTC AG and 1492r : 5'-TAC GGC TAC CTT GTT ACG ACTT) (Bioneer Corporation, Daejeon). PCR conditions used for amplification were: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1.5 min and a final extension at 72°C for 10 min. Reaction mixtures of 20 μL in total contained 2 μL of 10x PCR buffer, 2 μL of dNTP mix (2.5 mM each), 1 μL of each primer (10 pmoles), 50 ng of DNA template, 0.5 μL of i-Taq™ DNA polymerase (5U μL^{-1}) (iNtRON Biotechnology, Seongnam). The PCR products of approximately 1.4 Kbp were examined by electrophoresis in a 1.5% agarose gel. The PCR product was purified using PCRquick-spin™ (iNtRON Biotechnology, Seongnam). The 16S rRNA gene sequencing was done by Macrogen Inc. (Seoul) which uses ABI 3730xl DNA analyzer. The 16S rRNA sequences obtained were compared with the NCBI database using Blastn. Identity of ≥98% was the criterion used to identify the microbial species and strain.

Antimicrobial tests: Antimicrobial tests were performed based on the recommendation of the British Society for Antimicrobial Chemotherapy and National Committee for Clinical Laboratory Standards (2005) guidelines. Bacterial test cultures were grown overnight on Mueller Hinton broth (Becton, Dickinson and Company, Franklin Lakes). The inocula suspension concentration was diluted with 0.85% sterile saline solution to achieve an optical density between 0.08 to 0.1 units at 625 nm. Before inoculation, the inocula was diluted 10 times to make it approximately 10^8 colony forming unit per mL. Mueller Hinton agar plates were prepared in advance. Sterile cotton swabs were used to streak entire plates with the inoculum suspension. Sterile 6 mm filter paper discs (Whatman International Ltd, Maidstone) were used to place the samples on agar plates. The samples concentration was adjusted to 1 mg mL^{-1} for protein/peptide extract and 50 mg mL^{-1} for ethanolic extract. All the plates were incubated at 37°C for 16 h. The positive control used was Tetracycline (Oxoid, Basingstoke).

RESULTS AND DISCUSSION

Identification of test microorganisms: The microbes were identified using 16S RNA sequences as *Bacillus cereus* ATCC 14579, *Staphylococcus aureus* RF122, *Escherichia coli* UTI89 and *Pseudomonas aeruginosa.*

The results (Table 1, 2; Fig. 3) show the inhibition of various plant and fermented samples that were tested against gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*). The four bacteria obtained from the Microbiology Department were further analysed for their exact species/strain by 16S rRNA sequence determination and comparison to existing

Table 1: Inhibition zones of various plant and fermented ethanolic extracts

	Inhibition zone (mm) against			
Plant species/fermented samples	*E. coli*	*S. aureus*	*B. cereus*	*P. aeruginosa*
Andrographis paniculata	No inhibition	11±1.0	14±1.0	11.5±1.5
Curcuma mangga (Tumeric)	No inhibition	8.5±1.5	10.5±0.5	9.5±0
Carica papaya (Papaya leaf)	No inhibition	No inhibition	No inhibition	No inhibition
Allium sativum (Garlic)	No inhibition	No inhibition	7.0±1.0	7.0±1.0
Cymbopogon citratus (Lemon grass)	No inhibition	No inhibition	No inhibition	No inhibition
Fermented bean	No inhibition	No inhibition	No inhibition	7.0±1.0
Functional food paste	No inhibition	No inhibition	No inhibition	No inhibition
Fermented extract	No inhibition	No inhibition	No inhibition	8.0±1.0
Fermented vinegar with lactic acid bacteria	No inhibition	No inhibition	No inhibition	No inhibition
+ control	15±1.0	16±0	19±1.0	16.5±0.5
- control	No inhibition	No inhibition	No inhibition	No inhibition

All tests were done in triplicates

Table 2: Inhibition zone of various protein/peptide extracts

	Inhibition zone (mm) against			
Plant species/fermented samples	*E. coli*	*S. aureus*	*B. cereus*	*P. aeruginosa*
Andrographis paniculata	No inhibiton	No inhibiton	No inhibiton	No inhibiton
Curcuma mangga (Tumeric)	No inhibition	No inhibition	No inhibition	No inhibition
Carica papaya (Papaya leaf)	No inhibition	No inhibition	No inhibition	No inhibition
Allium sativum (Garlic)	15±1.5	28±2.1	16.3±1.5	9±0.9
Cymbopogon citratus (Lemon grass)	No inhibition	No inhibition	No inhibition	No inhibition
Zingiber officinale (Ginger)	No inhibition	No inhibition	No inhibition	No inhibition
Beta vulgaris (Beet root)	No inhibition	No inhibition	No inhibition	No inhibition
Allium cepa (Big onion)	No inhibition	No inhibition	No inhibition	No inhibition
Allium cepa (Small onion)	No inhibition	No inhibition	No inhibition	No inhibition
Momordica charantia (Bitter gourd)	No inhibition	No inhibition	9.0±0.3	10.0±0.9
Momordica charantia seeds	No inhibition	No inhibition	No inhibition	No inhibition
Agaricus bisporus stem (Button mushroom)	No inhibition	No inhibition	No inhibition	No inhibition
Agaricus bisporus fruiting body	No inhibition	No inhibition	No inhibition	No inhibition
+ ve Control	18±0.6	30±0	34±0	26±0
- ve Control	No inhibition	No inhibition	No inhibition	No inhibition

All tests were done in triplicates

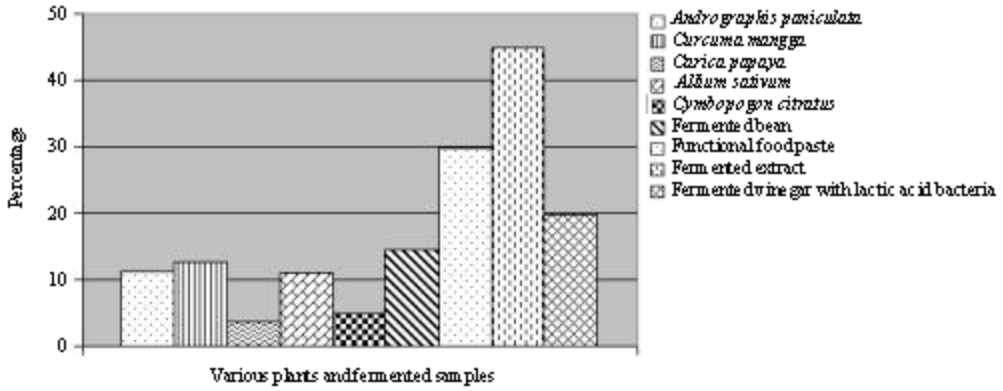

Fig. 1: Ethanolic extract yield. % (w/w) yield refers to gravimetric determination of total extractable compounds expressed as a percentage of the sample weight

databases. The amount of secondary metabolite and peptide extracted from various plant and fermented extracts are shown in the bar charts (Fig. 1, 2).

Fermented extract followed by functional food paste showed highest total extractable compounds from the ethanolic extract with 45 and 30%, respectively. Total extractable compound from fermented vinegar was 20% and the rest of the sample was below 20%. On the other hand, *Andrographis paniculata* and *Allium sativum* showed the highest protein content with 1.77 and 1.80 mg g^{-1}, respectively. These were followed by *Zingiber officinale* with 1.56 mg g^{-1}

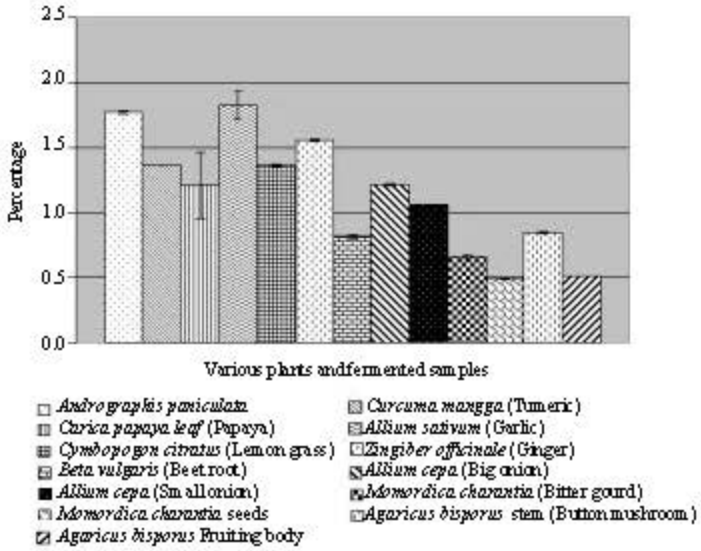

Fig. 2: Protein/peptide extract yield. Concentration refers to protein or peptide concentration in mg g^{-1} tissue

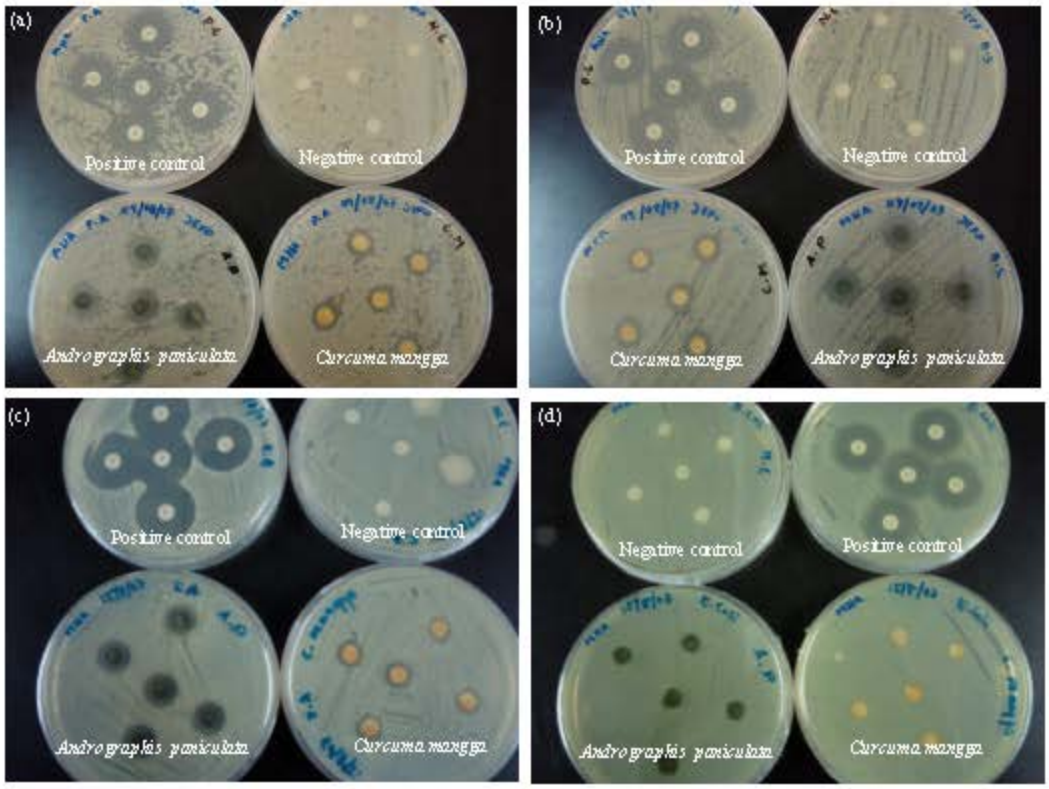

Fig. 3: Effect of ethanolic extracts on bacterial growth (a) *Pseudomonas aeruginosa*, (b) *Bacillus sereus*, (c) *Staphylococcus aureus* and (d) *E. coli*

and both *Curcuma mangga* and *Cymbopogon citratus* with 1.36 mg g^{-1}. The rest of the samples have protein content below 1.30 mg g^{-1}.

The most pronounced inhibition zone for ethanolic extract was obtained with *Andrographis paniculata* producing inhibition zones of 11 mm against *S. aureus*, 14 mm against *B. cereus* and 11.5 mm against *P. aeruginosa*. In studies done by Singha *et al.* (2003), the aqueous extract and the arabinogalactan protein fractions showed inhibition against *E. coli* and *P. aeruginosa* but

not towards *S. aureus*. Their 80% methanol and chloroform extraction of *Andrographis paniculata* did not show inhibition against *E. coli*, *P. aeruginosa* or *S. aureus*. Hence, these findings are contradictory to present findings. These differences could be attributed to the solvents used in the current study for extraction. Moreover, the used strain of the bacteria could also affect the results significantly.

Ethanolic extracts from *Curcuma mangga* produced inhibition zones of 8.5 mm against *S. aureus*, 10.5 mm against *B. cereus* and 9.5 mm against *P. aeruginosa*. Extracts from *Allium sativum* inhibits both *B. cereus* and *P. aeruginosa*, respectively with 7 mm inhibition zones. Ethanolic extracts prepared from fermented bean and fermented extract only inhibited *P. aeruginosa* with 7 and 8 mm inhibition zones, respectively. Ethanolic extracts from many plant sources have been shown to have biological activity against bacteria. For instance, the ethanolic extracts from *Rhus* (Nassar-Abbas and Halkman, 2004) are inhibitory towards gram positive and gram negative bacteria. A comprehensive review of the biological activities of *Rhus* extracts details the promising potential of the extracts of parts of this plant (Rayne and Mazza, 2007).

Peptide/protein extracts from *Allium sativum* showed promising results with 15 mm inhibition zones against *E. coli*, 28 mm against *S. aureus*, 16.3 mm against *B. cereus* and 9 mm against *P. aeruginosa*. Extracts from *Momordica charantia* were more selective and showed inhibition only against *B. cereus* and *P. aeruginosa* with 9 and 10 mm inhibition zones, respectively. Present results are in agreement with those of Gosh *et al*. (2008) who showed that aqeous extracts are generally less potent in their bioactivity than methanolic extracts.

CONCLUSION

Ethanolic extracts of *Andrographis paniculata* exhibited some degree of antibacterial activity towards *P. aeruginosa*, *S. aureus* and *B. cereus*. However, its peptide/protein extract did not produce any inhibition towards the bacteria species tested. Peptide/protein extract of *Allium sativum* exhibited a strong inhibition zone against both gram negative and gram positive bacteria but its ethanolic extract only produced a small degree of inhibition against *B. cereus* and *P. aeruginosa*. The particular compound responsible for the inhibition in each case is undergoing characterization by using High Performance Liquid Chromatography (HPLC) and mass spectrometry.

ACKNOWLEDGMENT

The researchers wish to thank University of Malaya for facilities and award of FP057/2005C grant to undertake this project.

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