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Full Length Research Paper

Identification and primary characterization of a plant antimicrobial peptide with remarkable inhibitory effects against antibiotic resistant bacteria

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On the basis of a primary screening scheme by using plant seed methanol extract against a collection of human pathogenic bacteria, *Medicago sativa* L. seeds were chosen as the best potential resource against tested Gram positive bacteria. Then an agar-overlay method using fully separated proteins on sodium dodecyl sulphate-polyacryliamide gel electrophoresis (SDS-PAGE) gels was used for initial determination and primary characterization of active putative defensins in the plant seeds. Clear and remarkable zones of inhibition in a region corresponding to peptides slightly larger than 6 kDa were recorded for Methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecium* (VRE) strains and yeast but a smaller inhibition zone with several colonies for tested Gram negative strain of *Escherichia coli*. Further characterization experiments using two-dimensional gel electrophoresis and subsequent agar-overlay assay against both strains confirmed the peptide nature of the active substance. Also gel filtration separation using Sephadex G-25 superfine and subsequent antibacterial assays could confirm the presence of a low molecular weight anti-MRSA and anti-VRE peptide in the total water soluble proteins obtained from *M. sativa* L. seeds which also showed high level of thermo stability.

Key words: *Medicago sativa* L., methicillin resistant bacteria, vancomycin resistant bacteria, plant defensins.

INTRODUCTION

Infections caused by multidrug resistant bacteria are major concern in any healthcare facilities. They raise the morbidity rate and cost of therapy according to prolonged hospital residence which could also increase the probability of resistant elements transmission (Wenzel and Edmond, 2001). *Stapylococcus aureus* is one of the most significant infectious agents in the community as well as in the hospitals (Wisplinghoff et al., 2004). A 62% increase in the *S. aureus* hospitalization, and more than 2 times increase in MRSA associated hospitalization in a 6 years period in the US, could reveal the importance of finding new and more powerful antibiotics (Klein et al., 2007). Vancomycin resistant *Enterococcus faecium* strains also are amongst the most important multidrug resistant human pathogens and cause great deal of progressively troubles in many healthcare systems (Deshpande et al., 2007; Hidron et al., 2007).

Plants are great and hopeful resource for achieving new antibacterial agents (Cowan, 1999; Thevissen et al., 2007). Furthermore, in the case of *S. aureus* there are many reports about screening of plant material for their anti - staphylococcal substances (Gibsson, 2004). The presence of some sophisticated d efence systems in

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Abbreviations: **SDS-PAGE**, Sodium dodecyl sulphate-polyacryliamide gel electrophoresis; **MRSA**, methicillin resistant *Staphylococcus aureus;* **VRE**, vancomycin resistant *Enterococcus faecium*.

plants which include the synthesis and secretion of some secondary metabolites with proofed antimicrobial properties, make them a good source for finding new antimicrobial agents, even for human pathogens (Cowan, 1999 and Thomma et al., 2003). Amongst different antimicrobial agents produced by these living organisms there are a number of antimicrobial peptides categorized as defensins. Plant defensins are very similar to animal defensins both in structure and function and could be a promising candidate for overcoming bacterial infections as well as fungal infections (Thomma et al., 2003; Thevissen et al., 2007). *Medicago sativa* L. belongs to Leguminoase and is a member of the genus Medicago with 83 different species. In the present study, this plant was chosen in the basis of data obtained from screening of several different methanol seed extracts (data not shown) regarding their antibacterial activities and according to the previously reported antibacterial and antifungal activities of plant materials obtained from different parts of this plant (Avato et al., 2006; Emmert et al., 1998; Gao et al., 2000). In our screening experiments using a collection of bacteria and yeast, *M. sativa* L. methanol extract demonstrated a remarkable and relatively uniform potential for inhibition of Gram positive bacteria and tested yeast but shown a very low activity against Gram negative bacteria. This is a characteristic of plant defensins that completely depends on their structure and their mode of actions (Thomma et al., 2003). The aim of this study was to evaluate *M. sativa* L. seeds as a potential source of plant defensins with probable application for combating with some human pathogenic bacteria.

MATERIALS AND METHODS

Microbial strains

Microbial strains included in this study were as follow: *Staphylococcus aureus* ATCC 25923, methicillin resistant *S. aureus* (MRSA) strain and vancomycin resistant strain of *E. faecium* (VRE) (kindly gifted by Professor M. M. Feizabadi), *Escherichia coli* ATCC 25922 and *Candida albicans* PTCC 5027.

Plant material and chemicals

M. sativa L. seeds were from Pakan Bazr, Isfahan Province, Iran, which its identity was confirmed using PCR experiments by the authors (data not shown). Organic solvents were from Caledon Laboratories Ltd (Ontario Canada). Reagents of polyacryl amide gel electrophoresis and gel filtration were from Sigma-Aldrich (St. Louis, MO).

Extraction of total water soluble protein from seeds and precipitation

Uniform seeds were selected, rinsed with water and distilled water and dried under a chemical hood at room temperature. Dried seeds were milled, after that total protein extraction was done by using a cold extraction buffer containing: 50 mM phosphate buffer pH 7, 2 mM EDTA, 5% glycerol and 50 mM NaCl. Cold extraction buffer was added to the milled seeds (10:1, V/W) and the mixture was shacked for 2 h at 4°C. Centrifugation was done at 12000 rpm for 20 min at 4°C and the clarified protein solution was passed through a sterile gauze tissue and then stored at -20°C. Ammonium sulphate was used for precipitation of protein components of mentioned crude extract (up to 80% saturation, overnight at 4°C) and precipitated proteins were collected by 20 min centrifugation at 12000 rpm.

Electrophoresis and molecular mass estimation

Protein concentration was determined by standard protocol (Bradford, 1976) and by using Bovine serum albumin (BSA) as standard. Thereafter 30 µg aliquots of crude seed total water soluble protein and precipitated proteins were mixed with 5 µl of sample buffer containing 2-mercaptoethanol as reducing agent, and heated for 3 min at 100°C and then run onto 15% SDS-PAGE with constant voltage of 100 V. Each electrophoresis experiment was terminated when the tracking dye was reaching 1 cm above the sealing part of the gel cast. Insulin was used as protein marker. Regarding the low resolution of low molecular weight protein and peptides in SDS-PAGE experiments, Tricine-SDS gels were used for better estimation of the molecular weight of the effective substance and for the determination of the amount of effective peptides by densitometer (Schagger and Von Jagow, 1987). Coomassie brilliant blue staining method was used for visualisation of protein bands as described elsewhere.

Agar-overlay assay

After electrophoresis of crud or precipitated materials of seed proteins, gels were subjected to the agar overlay assay as described previously (Ko and Ahn, 2000). Briefly, each gel slice was fixed initially by 30 ml of 20% isopropanol, 10% acetic acid in Milli-Q water for 2 h, and then was washed by 200 ml of Milli-Q water for additional 4 h, at room temperature. Each washed gel was placed on a sterile Petri dish and 7 ml of 50°C Mueller-Hinton medium containing 0.75% agar and 5×10^7 CFU of the test microorganism was poured on the gel. In the case of *Candida albicans*, potato-dextrose agar containing 0.75% agar was used and 2% NaCl (w/v) was added to MHA, for MRSA inhibition assay. Incubation was done at 35 and 30°C for 20 h for bacteria and yeast respectively. A SDS gel also was run with 25 µg samples of bovine serum albumin, insulin and water as a negative control and for investigation of the probable effect of sample buffer components in the antimicrobial assays for each tested microorganism. Both strains of *S. aureus*, VRE strain and strains of *E. coli* and *C. albicans* were subjected to agar-overlay assay.

Two-dimensional gel electrophoresis (2-DE) and subsequent agar overlay assay

For the characterization of effective antibacterial peptides, a protein sample was subjected for two-dimensional gel electrophoresis studies. Duplicate 2-DE gels were electrophoresed simultaneously, one for agar overlay assay and the other for silver nitrate staining to correlate antibacterial activity with peptide spots in silver nitrate stained gel. Protein samples were solubilised in a solution of 9 M urea, 1% DTT, 4% Chaps, 1% ampholyte (pH 3 to 10), and 35 mM Tris–HCl (pH 7 to 4). For analytical and preparative gels, 80 µg protein solutions were mixed with the aliquot rehydration buffer containing 8 M urea, 2% Chaps, 7% DTT, 0.28% ampholyte (pH 3 to 10) and a trace amount of bromophenol blue, respectively. The

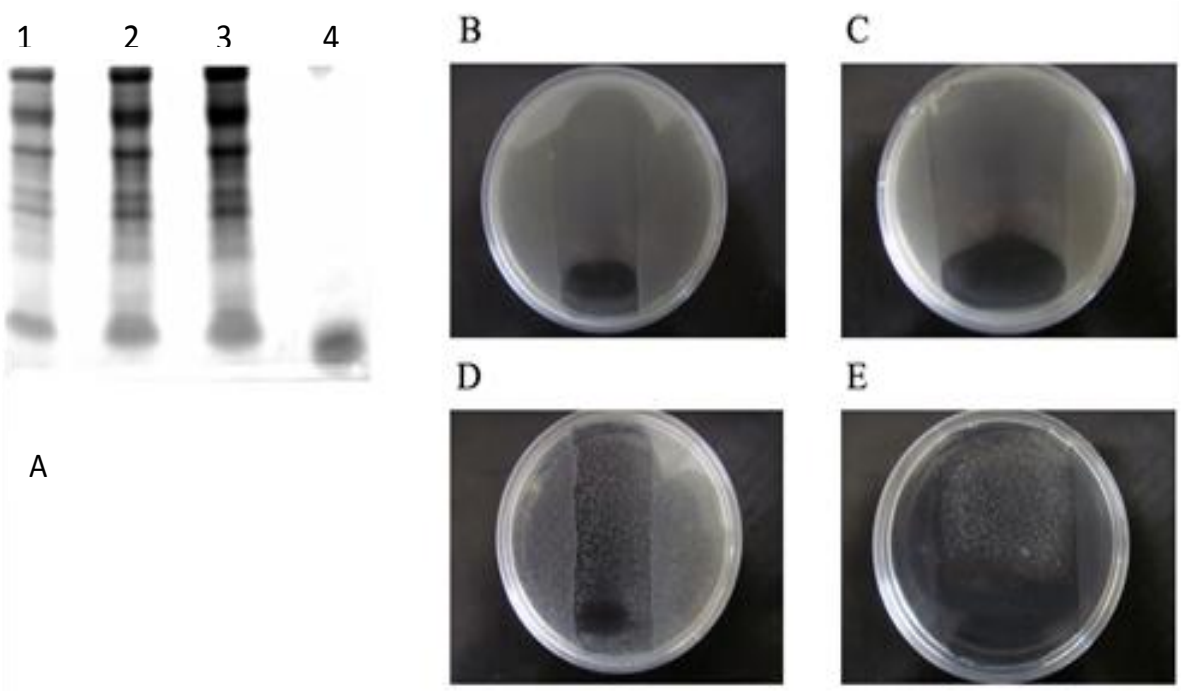

Figure 1. SDS-PAGE analysis and agar overlay assays against two antibiotic resistant bacterial strains. A. Left to right, Lanes 1-3: 25, 50 and 100 µg total water soluble proteins respectively; lane 4, insulin as low molecular weight protein marker. B and C: Anti-MRSA activities of *M. sativa* L. total water soluble proteins in agar overlay assays, when about 3 µg (B) or 10 µg (C) of peptides were assessed. D and E: Anti-VRE activities of *M. sativa* L. total water soluble proteins in agar overlay assays, when about 3 µg (D) or 10 µg (E) of peptides were assessed.

IPG strips (18 cm, pH 4 to 7, linear) were loaded with sample proteins during rehydration for 16 h at room temperature in re-swelling tray (Amersham Pharmacia Biotech, Uppsala, Sweden). IEF was conducted with a Multi phore II system (Amersham Pharmacia Biotech) for a total of 70 000 Vh. Thereafter, the strips were equilibrated for 15 min in a buffer containing 15 mM Tris-HCl.pH 8.8, 6 M urea, 87% glycerol, 2% SDS, bromophenol blue. IPG strips were applied on 12.5% SDS-PAGE gels at using a PROTEAN II Multi Cell (BioRad). The application voltage was 15 mA for an hour and continued with 35 mA to the end of the electrophoresis. The analytical gels were immediately scanned using GS-800 calibrated densitometer (BioRad) at 600 dpi resolution.

Gel filtration experiment for the separation of active antibacterial peptides

Sephadex G-25 superfine was used for the separation of effective peptides. An ammonium sulphate precipitated sample of total water soluble proteins was dissolved in sterile 10 mM Tris buffer (pH 7.2) and clarified by centrifugation at 12000 rpm, for 20 min at 4°C. After that the sample was dialysed against the same buffer using a 1 kDa dialysis tube and then applied onto an equilibrated packed column. Washing was done by 3 folds bed volume of the same sterilized buffer at flow rate of 1 ml/min. 2 ml fractions were collected and checked by Bradford reagent for determination of protein concentration. SDS-PAGE analysis could reveal those fractions containing low molecular weight protein and peptides.

Antimicrobial assay

Active antibacterial peptides were determined by broth microdilution susceptibility tests, where peptide containing fractions were assessed against MRSA and VRE strains. 10 mM Tris buffer (pH 7.2) also was assessed. The amounts of effective peptides were estimated by Bradford assay and a panel with final concentration range of 64 to 0.06 µg/ml of peptides was included in the study. Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined as recommended by Manual of Clinical Microbiology (9th edition) (Murray et al., 2007). In brief, 18 h cultures of each tested microorganism were used in susceptibility tests. A 2-fold serial dilution of each fraction (128 to 0.125 µg/ml) was prepared by sterile Mueller Hinton Broth medium in a 96 well trays. Equal volumes of bacterial suspensions were added to each well to final bacterial cell counts of 5×10^5CFU/ml. Inoculated trays were incubated for 20 h at 37°C. Results were recorded as the lowest concentration that could inhibit visible growth of microorganisms. MBCs were determined by sub-culturing of 100 µl of each negative well onto a NA plates. The results were recorded after 24 h incubation at appropriate temperatures, as the lowest concentrations that could kill 99.99% of the initial microorganisms counted in quality control step as recommended elsewhere (Murray et al., 2007). All experiments were done in triplicate and chloramphenicol was used as standard antibiotic.

RESULTS

Figure 1A demonstrates the total water soluble proteins stained by Coomassie brilliant blue reagent after running onto a 15% polyacryl amide gel.The molecular weight of our desired active peptides was estimated to be between 6 to 10 kDa according to the data resulted from agar-

overlay assays.

When 50 µg/well total proteins (crude or precipitated by using ammonium sulphate) were used in agar overlay assays, there were clear zones of inhibition near the end of run gels for three Gram positive bacteria and yeast but with several small colonies in the case of tested *E. coli* strain. According to data resulted from 3 fully separated experiments (including total water soluble protein preparation, SDS-PAGE and subsequent agar overlay assays), the size of inhibition zones were varying for different bacteria and was as follow: *S. aureus*> VR *E. faecium* > MRSA> *C. albicans.* For MRSA and VR *E. faecium* strains, we also repeat the assay with different amount of total protein as shown in Figure1B, C, D and E respectively. Corresponding amounts of effective peptides were estimated to be about 3 and 10 µg by densitometer according to the concentration of total samples loaded onto each well. Neither BSA nor insulin resulted in any inhibitory zone of growth in agar overlay when tested against bacteria and yeast.

Silver nitrate stained two-dimensional gel and its correspond agar overlay are shown in Figure 2. After agar overlay assay, there was a clear zone of inhibition of tested MRSA strain in that part of gel which corresponds to a cationic peptide with isoelectric point of about 5. Resazurin stained gels also could confirm the absence of growth of bacterium in that part of gel.

Antibacterial susceptibility tests were also carried out with peptide containing fractions of gel filtration experiments and showed a MIC of 4 µg/ml for MRSA and 1 µg/ml for VRE tested strains in triplicate experiments. The antibacterial activities were maintained when heat treatment of the most active fraction were carried out up to 8 min at 80°C water bath. MICs for chloramphenicol (as standard antibiotic) were 16 and 4 µg/ml when tested against MRSA and VRE strains respectively.

DISCUSSION

Antimicrobial peptides are widespread among diverse range of living organisms, from bacteria and fungi to insects, plants and animals (Hara et al., 2008; Thomma et al., 2003). These antimicrobial substances increasingly gain more attention regarding of the importance of combating with new generations of antibiotic resistant bacteria (Hancock et al., 2006). One of the most important advantages of antimicrobial peptides is that in contrast to conventional antibiotics, they have several targets and several modes of action simultaneously. So resistance against such antibacterial substances is apparently more difficult to be emerged in comparison with existing antibiotics (Hancock et al., 2006). However, some human pathogenic bacteria had been able to develop resistance against human antimicrobial peptides during evolution (Peschel, 2006). So plant antimicrobial peptides could be better than human one in this regard, because they have had no or rare contact with human pathogens to induce such resistance mechanisms in them.

Plant defensins are now going to be considered as an important source for new antibiotic discovery and designing. They are considered as just antifungal substances for a long time and as a tool for combating with just plant pathogenic fungi for a long time (Gao et al., 2000; Wang and Ng, 2007).

In this study, we investigated the probability of presence of antibacterial peptides in *M. sativa* L. seeds. This plant seed was chosen according to data obtained from initial antimicrobial screening experiments by methanol extracts of 10 different plant seeds against 12 different microorganisms (data not shown). Plant antimicrobial peptides are very similar to other antimicrobial peptides regarding their greater activities against Gram positive bacteria (Thomma et al., 2003; Peschel and Sahl, 2006; Portieles et al., 2006). *M. sativa* L. has some antimicrobial substances such as saponins (Avato et al., 2006), canavanine (Emmert et al., 1998) and some important antifungal defensins (Gao et al., 2000).

In comparison to other reported studies on identification and characterization of plant defensins, our aim could be achieved easier and faster. Most reported studies have been conducted via several chromatographic steps (Wang and Ng, 2007; Odintsova et al., 2008). We used a simple agar-overlay assay which could show any antimicrobial proteins present in the water soluble proteins of seeds.

2-dimensional gel electrophoresis and subsequent agar overlay assays could discover the most effective anti-MRSA and anti-VRE peptide as well. There were also a few other very small and unclear zone of inhibition in agar overlay assays which correspond to some peptides and even proteins but the most significant effect was seen around that spot indicated in Figure 2. According to the data obtained from this part of the study, the effective substance is a cationic peptide with IP of about 5 and molecular mass of about 6 to 10 kDa. The molecular mass of antibacterial peptide also was confirmed by gel filtration experiments and subsequent antibacterial susceptibility tests.

Regarding to the results of our study, we concluded that there is a very effective antibacterial peptide in *M. sativa* L. seeds with great activities against our MRSA and vancomycin resistant strain of *E. faecium*. It may be possible to be a synergistic effect between this peptide with other antibacterial peptides as indicated in agar-overlay assays which carried out by non-denaturing gels (data not shown).

On the basis of the results of heat treatment experiments, our antibacterial peptide has one of the most important characteristic of defensins; thermo stability, which in combination with its IP (~5) could confirmed it as a defensin with great activity against two important pathogenic bacteria.

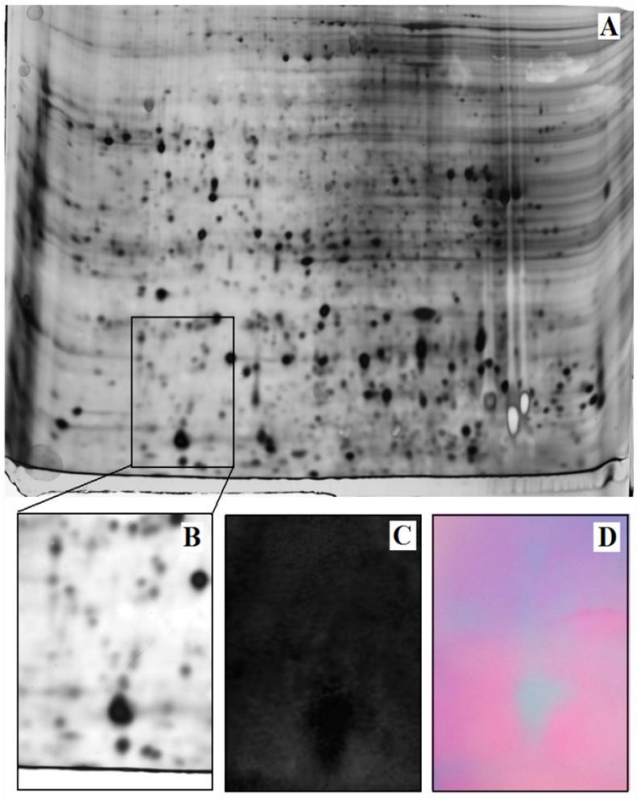

Figure 2. Identification of effective anti- MRSA peptide by using 2-dimensional gel electrophoresis and subsequent agar overlay assay. A. Silver nitrate stained gel. B. Effective peptides in the stained gel. C and D, Inhibitory zone in agar overlay assay against MRSA strain before and after staining by resazurin reagent. The arrow shows the direction of pH gradient (from pH 4 to 7).

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REFERENCES

Avato P, Bucci R, Tava A, Vitali C, Rosato A, Bialy B, Jurzysta M (2006). Antimicrobial activity of saponins from *Medicago* sp.: Structure-activity relationship. Phytother. Res. 20: 454-457.

Bradford MM (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564582.

Deshpande LM, Fritsche TR, Moet GJ, Biedenbach DJ, Jones RN (2007). Antimicrobial resistance and molecular epidemiology of vancomycin-resistant enterococci from North America and Europe: a report from the SENTRY antimicrobial surveillance program. Diagn. Microbiol. Infect Dis. 58: 163-170.

Emmert EAB, Milner JL, Lee JC, Pulvermacher KL, Olivares HA, Clardy J, Handelsman J (1998). Effect of canavanine from alfaalfa seeds on the population biology of *Bacillus cereus*. Appl. Environ. Microbiol. 64(12): 4683-4688.

Gao A, Hakimi SM, Mittanck CA, Wu Y, Woerner MB, Stark DM, Shah DM, Liang J, Rommens CMT (2000). Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat. Biotechnol. 18: 1307-1310.

Gibbson S (2004). Anti-staphylococcal plant natural products. Nat. Prod. Rep. 21: 263-277.

Hancock REW, Sahl H-G (2006). Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. 24(12): 1551-1557.

Hara s, Mukae H, Sakamoto N, Ishimoto H, Amenomori m, Fujita H, Ishimatsu Y, yanagihara K, Kohno S (2008). Plectasin has antibacterial activity and no affect on cell viability or IL-8 production. Biochem. Biophys. Res. Commun. 374: 709-713.

Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK (2007). Antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007". Infect. Control. Hosp. Epidemiol. 29(11): 996-1011.