Pak. J. Bot., 43(6): 2971-2978, 2011.

ISOLATION AND CHARACTERIZATION OF PEPTIDE(S) FROM *PISUM SATIVUM* HAVING ANTIMICROBIAL ACTIVITY AGAINST VARIOUS BACTERIA

SAIMA REHMAN AND AZRA KHANUM

Department of Biochemistry, PMAS-Arid Agriculture University Rawalpindi, Pakistan
Corresponding author email: azrakhanum@uaar.edu.pk

Abstract

A systematic approach was taken to isolate and characterize the antimicrobial peptide(s) from the crude aqueous extract, solubilized ammonium sulphate precipitates and purified gel filtration chromatographic fractions of seed/pod of *Pisum sativum* L. (garden pea). Their antibacterial activity was investigated against a number of bacteria: *Micrococcus luteus, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Proteus vulgaris Pasterurella multocida, and Pseudomonas aeruginosa* using disc diffusion method. Two active peptides from seed i.e., S_4, S_5 and pod i.e., P_7, P_8 were obtained having molecular weight ~19 kDa, ~22 kDa, ~10 kDa and ~11 kDa, respectively. The bioactivity of each peptide was tested against different enzymes, temperatures and pH. The results showed that the all purified peptides were susceptible to inactivation by trypsin and proteinase K, stable at temperature 4, 25°C and active at pH 5-7. Further *S. aureus* was found to be the most sensitive strain based on minimum inhibition concentration (MIC) value.

Introduction

From ancient time, plants have been a precious source of natural products for sustaining human health. According to World Health Organization (Anon., 2008) more than 80 % of the world's population relies on traditional medicine for their primary healthcare needs. Therefore medicinal plants need to be investigated for better understanding in respect to their properties, safety and efficiency. One of such properties like antimicrobial activity can be of great significance in therapeutic treatments. Especially with the emergence of antibiotic-resistant microbes which have become a major health threat over the past decades (Wright, 2000). According to Marx (2005), the continuous use of analogous antibiotics has resulted in multi-resistant bacterial strains all over the world. Presently it is being broadly predictable that in the very near future antibiotic resistance will make healthcare professionals helpless toward effective therapies for bacterial infections. Consequently there is an urgent need to search for unconventional antibiotics. Therefore as an upshot, interest in peptide antibiotics has increased greatly during the past decade, as these are believed to be very potent, showing higher activity, higher specificity, having few toxicology problems, do not accumulate in organs, few drug-drug interaction challenges and are biological and chemical diverse.

It has been shown in certain studies that plants protect themselves against microbial pathogens by various defense responses including production of antimicrobial peptides (AMPs), secondary metabolites, lytic enzymes and membrane-interacting proteins (Feng *et al*., 2003). The fact that the common features for most peptides are i) a net positive charge and ii) an amphipathic nature which allows them to persist at water-lipid interfaces and then to disturb microbial membrane components (Ruissen *et al*., 2001). Peptides have come a long way from the time of development to their use in therapeutics. The peptide therapeutics market is providing new commercial opportunity to biotechnology and pharmaceutical industries (http://www.mindbranch.com). Similarly advances in peptide synthesis and high-throughput activity screening have made possible the *de novo* and rational design of novel peptides with improved properties (Marcos *et al*., 2008).

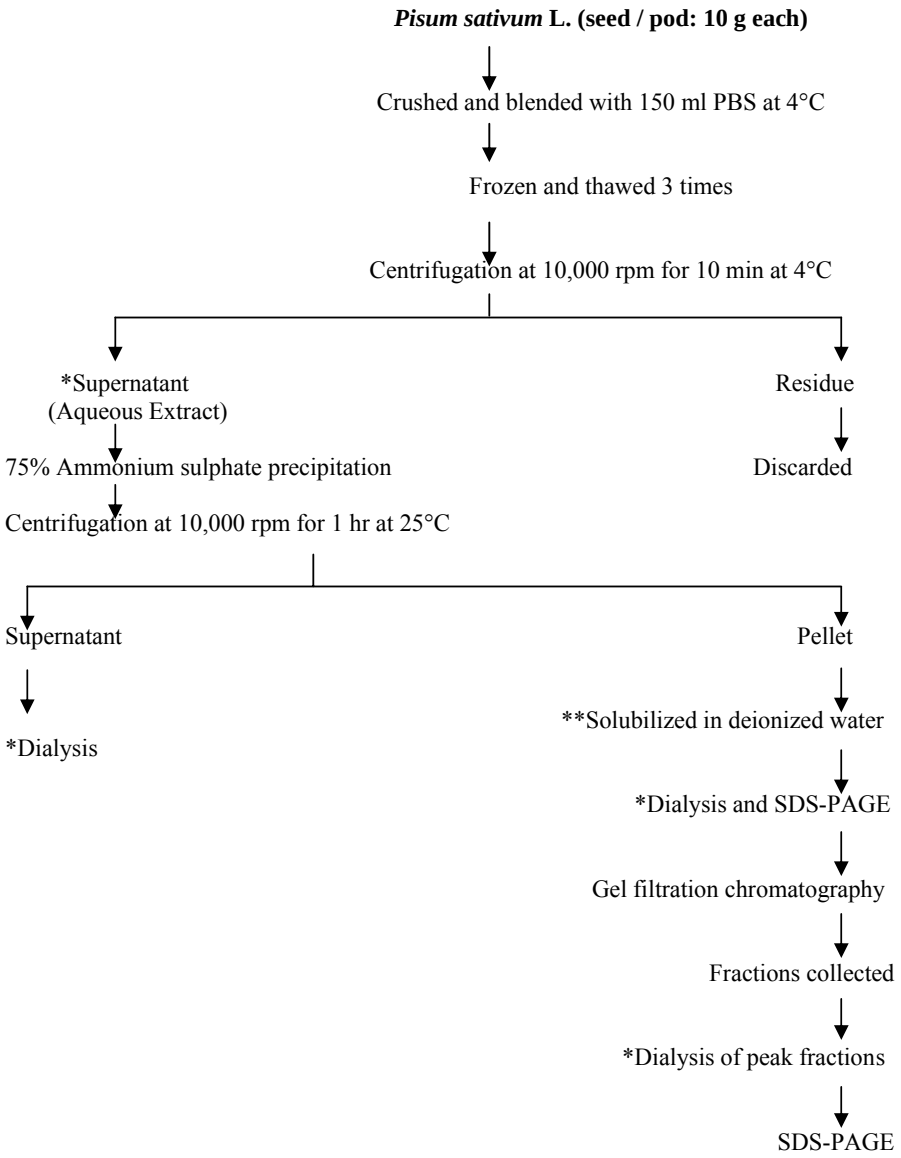
Previous studies have reported many antimicrobial peptides isolation from common vegetables and spices (Ngai & Ng, 2004; Hu *et al*., 2004; Oard *et al*., 2004; Talas, 2004; Xia and Ng, 2005; Ngai *et al*., 2005; Mariângela *et al*., 2006). *Pisum sativum* L (garden pea) belongs to family Fabaceae, one of the biggest family of dicot plants and is a source of medicinal plants used in the allopathic and traditional medicines. Pea seeds are eaten cooked and uncooked as a vegetable and are marketed fresh, canned or frozen while ripe dried peas are used as whole, split or made into flour (Davies *et al.,* 1985). The protein concentration of peas ranges from 15.5-39.7% (Muehlbauer & Tullu, 1998). Like all vegetable protein, its amino profile is deficient in some essential amino acids, for example cysteine and methionine. Pod is used in the world of beauty packs, herbal plasters and in folk cures for fungus infections due to the presence of the small amounts of digestive enzymes having skin softening effect. The young pods have a sweet flavor but there is only a thin layer of flesh with a fibrous layer beneath it. Flour made from dried pods is energy rich (www.iron-clay.compea.html). Keeping in view the medicinal values of *P. sativum* and therapeutic importance of peptides, present study was carried out in an effort to make beneficial use of seed and specially pod which is thrown out as a waste. Therefore AMPs seed and pod of *P. sativum* having antibacterial activity were purified and characterized. Further stability of AMPs was checked using different enzymes, temperature, pH and minimal inhibitory concentration (MIC).

Materials and Methods

Microorganisms and media: Cultures of various bacteria were obtained from Depository of Biotechnology Laboratory, PMAS Arid Agriculture University Rawalpindi to be used for the determination of antimicrobial activity. These microbes include *Micrococcus luteus* (*M. luteus*) *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Escherichia coli* (*E. coli*), *Klebsella pneumonia* (*K. pneumonia*), *Salmonella typhi* (*S. typhi*), *Proteus valgarcus* (*P. valgarcus*), *Pasterurella multocida* (*P. multocida*) *and Pseudomonas aeruginosa* (*P. aeruginosa*). Lauria Bertaini (LB) media was used for culturing above mentioned bacteria.

Protein extraction: *Pisum sativum* L. was collected from the local markets of Rawalpindi city, Pakistan. The strategy used for isolation, purification, characterization and determination of antimicrobial activity from seed and pod is given in Flow chart 1.

Chromatographic profiling, SDS-PAGE and minimum inhibition concentration (MIC): The dialyzed samples of both solubilized ammonium sulphate precipitates and supernatant were first used for estimation of antimicrobial activity. Since activity was observed only in solubilized not in supernatant of ammonium sulphate precipitates therefore, 2 ml having ~500 µg / ml protein concentration of this sample was applied onto the 1.5 x 70 cm column of Sephadex G-100, pre-equilibrated with 0.02M Sodium acetate buffer (pH 4.5).



Flow chart 1. Strategy for the isolation of antimicrobial peptide from *Pisum sativum* L. seed / pod.
*Protein estimation and zone inhibition assay were carried out. **Protein estimation only.

The column was eluted with the same buffer at the rate of 1ml/1min. The elution pattern was monitored by taking absorbance of collected fractions at 280 nm. All peak fractions were further subjected to check their antimicrobial activity. The fractions having antimicrobial activity along with crude extract and solubilized ammonium sulphate precipitate were run on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% and 14% gel for seed and pod samples respectively, by method described by Laemmli (1970). The gels were stained with coomassie brilliant blue R-250 and distained with methanol: acetic acid: water (30:60:10 v / v). The approximate molecular weight of fractions having antimicrobial activity was determined by plotting R_f versus molecular weight of known standard proteins.

Protein concentrations in crude extract, solubilized ammonium sulphate precipitates, supernatant of ammonium sulphate precipitate and fractions of gel filtration chromatography were estimated by using Bradford method (Bradford, 1976). The determined protein concentration in crude extract of seed was 835µg/ml, in solubilized and dialyzed ammonium sulphate precipitates was 635µg/ml, in

peak fraction S_4 was 200µg/ml and in S_5 was 145µg/ml. Similarly, the determined protein concentration in crude extract of pod was 754 µg / ml, in solubilized ammonium sulphate precipitates was 205µg/ml, in peak fraction P_7 was 30µg/ml and in P_8 was 40µg/ml.

Antibacterial assay: The antimicrobial activity of crude extract, solubilized ammonium sulphate precipitates, supernatant of ammonium sulphate precipitates and fractions of gel chromatography was determined using disc diffusion method (Bauer et al., 1966). Sterile Whatman filter paper discs of 6 mm in diameter were soaked with 20 µg/ 30 µl sample and placed on the surface of the agar plate. The antibiotic chloramphenicol was used as a control. These plates were incubated over night at 37°C. The results expressed as the mean ± SEM, indicate the standard deviation of the triplicate incubations in millimeter (mm). Excel statistical software was used to analyze the data (Borchardt *et al*., 2008).

Macro dilution assay was performed to establish minimum inhibition concentration (MIC) values of isolated peptides against *E. coli, S. aureus*, *S. epidermidis* and *S. typhi* as described by Wang (2003). The 10^8 CFU/ml test cultures were inoculated into LB broth containing 0-200 µg/ml antimicrobial protein preparation.

Enzymatic, temperature and pH stability: Two protein enzymes proteinase K (Fermentas, USA Cat # EO0491) and trypsin (Sigma, USA Cat # T 7409) were used to check their effect on antimicrobial activity of purified peptide(s) using method of Carol *et al*., (2005). Purified peptide samples of 3-20µg/10µl were mixed in 0.02 M Tris-HCl, pH 6.8 and then 1-2 U/2µl of enzyme was added into the mixture having final reaction volume 100µl and kept for 5 minutes. The activity of enzyme was stopped by heating the solution in thermo mixer for 5 minutes. The effect of various temperature and pH was also checked on antimicrobial activity of purified peptide(s). For this purpose 0, 4, 25, 50°C and pH; 4, 5, 6, 7 and 8 were used. Those samples which resisted up to 50°C, their activity were further checked at 70°C and 90°C. The purified peptides mixed in 0.02M Tris-HCl, pH 6.8 were heated to different temperatures for 10 minutes and then bioassayed. Similarly the pH stability of purified peptides was determined between the range of pH 4-8 using 0.02M sodium acetate and Tris-HCl as buffer solution.

Results and Discussion

Antimicrobial activity in crude extract: A systematic approach was used in this study to isolate and characterize potential peptide(s) having antimicrobial activity against various bacteria. The seed/pod of *Pisum sativum* was used to prepare crude aqueous extract. Antimicrobial activity of crude extract was investigated on LB agar plates against various bacteria in triplicate i.e., *M. luteus, S. aureus, S. epidermidis, E. coli, K. pneumonia, S. typhi, P. vulgaris, P. multocida* and *P. aeruginosa.*. Highest antimicrobial activity was found against *S. aureus* followed by *S. typhi, E. coli, M. luteus* and *S. epidermidis* while low activity was observed against *P. vulgaris, P. aeruginosa,* followed by *K. pneumonia* and *P. multocida* (Table 1). Similarly, crude extract of pod showed highest activity against *S. aureus* followed by *E. coli, S. epidermidis*, *S. typhi*, *M. luteus, K. pneumonia* and *P. aeruginosa*. No activity was observed against *P. vulgaris and P. multocida* (Table 2).

Antimicrobial activity in solubilized and supernatant of ammonium sulphate precipitates: The solubilized ammonium sulphate precipitates of seed/pod showed various level of antimicrobial activity while supernatant of ammonium sulphate precipitates did not show any activity. Solubilized ammonium sulphate precipitates of seed showed highest activity against *S. aureus* followed by *S. typhi*, *E. coli* then *S. epidermidis, K. pneumonia, M. luteus, P. aeruginosa* and low avtivity against *P. vulgaris* and zero activity against *P. multocida* (Table 1). Similarly solubilized ammonium sulphate precipitates of pod exhibited highest activity against *S. aureus* followed by *S. epidermidis*, *E. coli, S. typhi* then *M. luteus* and low avtivity against *K. pneumonia* and *P. aeruginosa* while zero activity against *P. vulgaris* and *P. multocida* (Table 2).

Purification of peptides: After the determination of antimicrobial activity, the solubilized ammonium sulphate precipitates of seed/pod were then subjected to the gel filtration column chromatography using Sephadex G-100. The separation profiles are shown in Figs. 1 & 2. In case of seed, total 5 peaks were obtained ranging from high molecular weight to low molecular weight protein(s) / peptide(s) which were designated as S_1 to S_5. All peak fractions were then checked separately for antimicrobial activity. Fractions S_4 and S_5 showed activity against various bacteria as shown in Table 1. The clear zone against *S. aureus* of peptides S_4 and S_5 was found to be doubled as compared to the clear zone of chloramphenicol which was used as control. Similarly double clear zone was found against *E.coli* as compared to clear zone formed by control. In case of pod, total eight peaks were obtained ranging from high molecular weight to low molecular weight protein(s)/peptide(s) which were designated as P_1 to P_8. All peak fractions were checked separately for antimicrobial activity. Peak fraction P_1. P_6 did not show activity against any bacteria used whereas peak fractions P_7 and P_8 showed activity against various bacteria. The broadest clear zone by P_7 fraction was found against *S. aureus* and *S. typhi* as compared to the clear zone of chloramphenicol and very small clear zone in case of *M. luteus* and *K. pneumonia* whereas in case of P_8 fraction, the broadest clear zone was observed against *S. typhi* followed by *E. coli* as compared to the clear zone of chloramphenicol.

SDS–PAGE: The crude extract, solubilized ammonium sulphate precipitates and purified fractions (S_4 and S_5) of seed were subjected to 12 % SDS-PAGE along with molecular weight markers ranging between 14.4 -116.0 kDa.. The pattern of protein separation on SDS-PAGE was found to be same in samples of crude extract and solubilized ammonium sulphate precipitates while purified peptides obtained by gel filtration chromatography as peaks 4 and 5 appeared as a single band in each with a molecular weight of ~19 kDa and ~22 kDa. The result is shown in Fig. 3. Similarly the crude extract, solubilized ammonium sulphate precipitates and purified fractions (P_7 and P_8) of pod were subjected to 14 % SDS-PAGE along with molecular weight markers ranging between 10 -170 kDa. The pattern of protein separation on SDS-PAGE was found to be same in samples of crude extract and solubilized ammonium sulphate precipitates while purified peptides obtained by gel filtration chromatography as peaks 7 and 8 appeared as a single band in each with a molecular weight of ~11 kDa and ~12 kDa. The result is shown in Fig. 4.

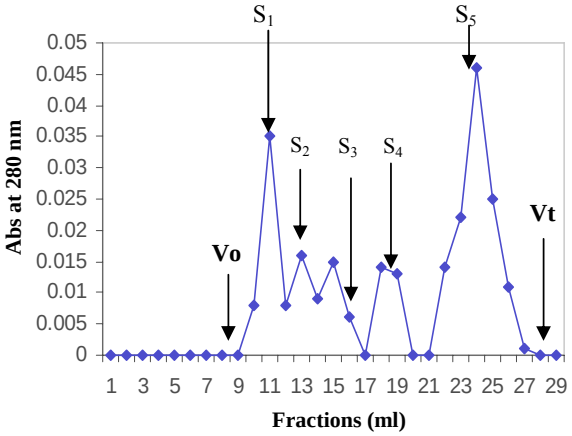


Fig. 1. Gel filtration chromatography of solubilized ammonium sulfate precipitates from seed of *Pisum sativum*. Arrows showing peaks 1-5.

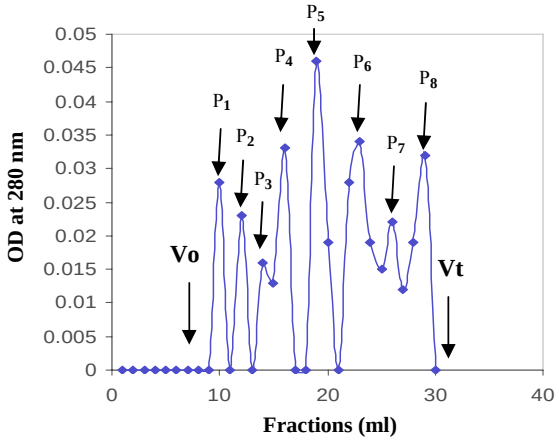


Fig. 2. Gel filtration chromatography of solubilized ammonium sulfate precipitates from pod of *Pisum sativum*. Arrows showing peaks 1-8.

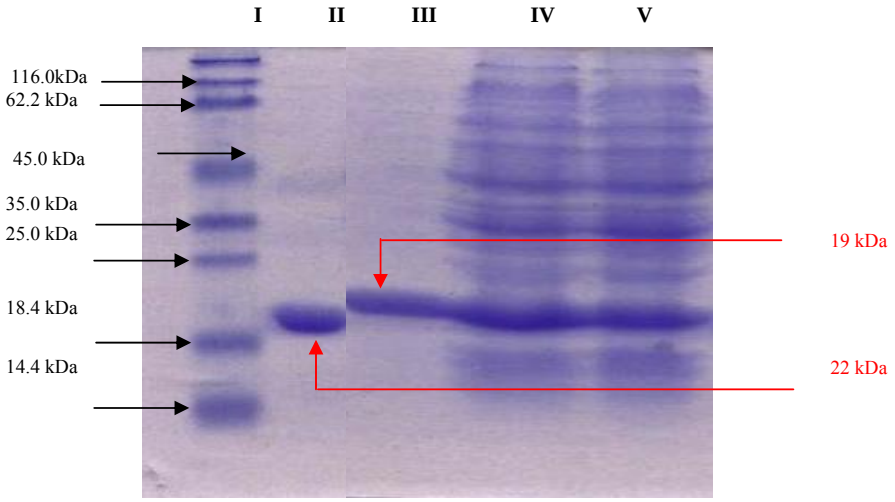


Fig. 3. SDS-PAGE of protein samples of seed of *Pisum sativum* L. Lane I, Molecular weight marker # 0431; Lane II, Purified peptide fraction (S_5) of GFC; Lane III, Purified peptide fraction (S_4) of GFC; Lane IV, Crude extract of seed; Lane V, Solubilized ammonium sulfate precipitates.

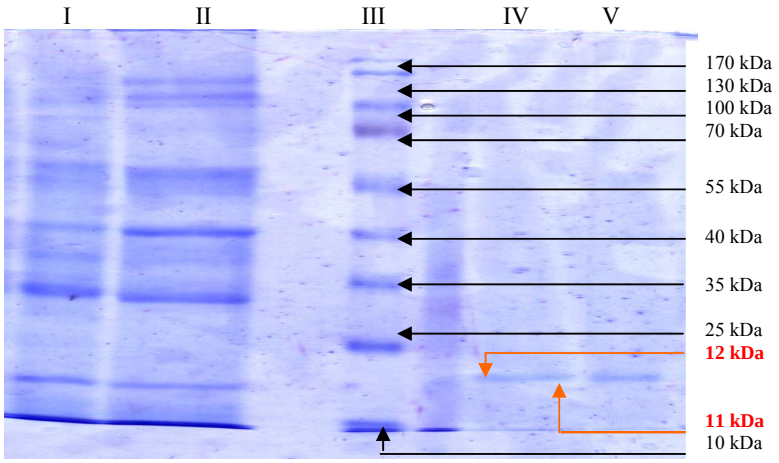


Fig. 4. SDS-PAGE of protein samples of pod of *Pisum sativum* L. Lane I, Solubilized ammonium sulfate precipitates; Lane II, Crude extract of pod; Lane III, Molecular weight marker # SM 0671; Lane IV, Purified peptide fraction (P_7) of GFC; Lane V, Purified peptide fraction (P_8) of GFC.

Table. 1. Antimicrobial activity of different samples of *P. sativum* seeds against various bacteria. Cam [34]- Chloramphenicol (34 mg / ml). *Not processed further because of low inhibitory activity.

Protein contents per disc (20 µg/30 µl)	Gram positive (clear zone in mm)			Gram negative (clear zone in mm)					
	M. luteus	*S. aureus*	*S. epidermidis*	*E. coli*	*K. pneumonia*	*S. typhi*	**P. vulgaris*	**P. multocida*	**P. aeruginosa*
Crude seed extract	3.20 ± 0.69	7.30 ± 0.115	3.20 ± 1.15	4.80 ± 0.17	0.80 ± 0.1	6.5 ±1.00	1.60 ± 0.288	0.80 ± 0.06	1.60 ± 0.51
Solubilized $NH_4(SO_4)_2$ precipitates	4.20 ± 0.11	10.50 ± 0.5	5.30 ± 1.0	8.40 ± 1.0	5.30 ± 0.5	8.50 ± 0.57	1.70 ± 00	0.00	4.20 ± 1.01
S_4 (Gel Chromatographic Fraction)	3.30 ± 0.23	46.70 ± 0.42	16.70 ± 0.58	33.30 ± 0.5	0.00	30.00 ± 0.58	-	-	-
S_5 (Gel Chromatographic Fraction)	0.00	41.90 ± 0.81	0.00	32.50 ± 0.29	0.00	4.60 ± 0.058	-	-	-
Cam[34]	15.00	24.00	23.00	14.00	15.00	14.00	9.00	14.00	14.00

Table. 2. Antimicrobial activity of different samples of *P. sativum* pods against various bacteria. *Not processed further because of low inhibitory activity.

Protein contents per disc (20 µg /30 µl)	*M. luteus*	*S. aureus*	*S. epidermidis*	*E. coli*	*K. pneumonia*	*S. typhi*	*P. vulgaris*	*P. multocida*	*P. aeruginosa*
Crude pod extract	4.4 ± 0.06	14.1 ± 0.17	6.2 ± 1.15	6.3 ± 0.35	3.1 ± 0.06	5.7 ± 0.21	0.00*	0.00*	2.6 ± 0.0
Solubilized $NH_4(SO_4)_2$ precipitates	13.1 ± 0.57	50.8 ± 1.0	36.1 ± 0.06	22.9 ± 0.06	9.8 ± 0.06	19.7 ± 0.11	0.00	0.00	9.8 ± 0.06
P_7 (Gel Chromatographic Fraction)	20 ± 0.0	120 ± 0.30	0.00	0.00	20 ± 0.1	120 ± 0.0	-	-	0.00
P_8 (Gel Chromatographic Fraction)	0.00	50 ± 0.06	16.6 ± 0.06	133 ± 0.20	33.3 ± 0.115	166.6 ± 0.36	-	-	16.6 ± 0.06
Cam[34]	15.00	30.00	20.00	14.00	17.00	16.00	9.00	14.00	15.00

Table. 3. Effect of temperature on the antimicrobial activity of purified peptides of seed (S_4, S_5) and pod (P_7, P_8) against various bacteria. Values are the mean ± SEM of triplicate determination from a representative experiment of the three experiments performed. Cam [34] Chloramphenicol (34 mg / ml).

Temp.	S_4 (6µg/30µl protein conc.)				S_5 (4.35µg/30µl protein conc.)				P_7 (0.9µg/30µl protein conc.)				P_8 (1.2µg/30µl protein conc.)			
	S. aureus	*S. epidermidis*	*E. coli*	*S. typhi*	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhi*	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhi*	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhi*
0 °C	2.0 ± 0.21	0.0	0.0	0.0	1.0 ± 0.0	-	0.0	1± 0.2	2.0 ± 0.0	-	-	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.06	0.0	2.± 0
4 °C	8.0±0.057	1.0±0.0	4.0 ± 0.0	3.0 ± 0.06	9.0 ± 0.0	-	4.0±0.06	4 ± 0	14.0 ± 0.06	-	-	13.0 ± 0.21	11 ± 0.017	4.0 ± 0.06	6.0 ± 0	4 ± 0.17
25 °C	1.0 ± 0.0	3.0 ± 0.06	9.0 ± 0	5.0 ± 0.06	3.0 ± 0.06	-	6.0 ± 0	4 ±0	14.0 ± 0	-	-	14.0 ± 0	12.0 ± 0	4.0 ± 0.21	7.0 ± 0	10 ± 0.21
50 °C	0.0	0.0	0.0	0.0	0.0	-	0.0	4 ± 0	0.0	-	-	2.0 ± 0	0.0	0.0	1.0 ± 0	14 ± 0
70 °C	-	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	-	0.0
90 °C	-	-	-	0.0	-	-	-	0.0	-	-	-	0.0	-	-	-	0.0
Cam [34]	24.0	16.0	17.0	19.0	24.0	-	17.0	19.0	24.0	-	-	19.0	24.0	16.0	17.0	19

Biochemical assays of AMPs

Effect of various temperatures: The effect of various temperatures on the antimicrobial activity of purified peptides of seed (S_4, S_5) and pod (P_7, P_8) were checked against *S. aureus, S. epidermidis, E. coli* and *S. typhi* because purified peptides had shown effectiveness against these microbes. All these peptides behaved differently at different temperatures against various bacteria. The results are compiled and presented in Table 3. Both the peptides S_4 and S_5 showed either zero or low activity at 0°C against all microbes used. Whereas at 4°C, S_4 and S_5 both showed highest activity against *S. aureus*, which was decreased at 25°C and completely lost at 50°C. Peptide S_4 showed highest activity at 25°C against *S. epidermidis, E. coli* and *S. typhi* while S_5 showed highest activity at 25°C only against *E. coli*, no activity against *S. epidermidis* and constant activity against *S. typhi* at 4°C, 25°C and 50°C except 0°C. Further S_4 and S_5 lost its activity against all microbes at 50°C, except the activity profile of S_5 against *S. typhi* because loss of activity occurred at 70°C. In case of pod peptides, P_7 and P_8 showed variable activity at different temperatures against various bacteria. Like S_4 and S_5, P_7 and P_8 showed the lowest activity at 0°C against all bacteria used and complete loss of activity at 50°C or 70°C. P_7 showed highest activity at temperature 4°C and 25°C while loss of activity at 50°C against *S. aureus* while no activity was observed against *S. epidermidis* and *E. coli* at all temperatures used. The activity of P_7 against *S. typhi* showed maximum activity at 25°C with decrease at 50°C and complete loss at 70°C. P_8 showed highest activity against *S. aureus* at 25°C then loss of activity at 50°C while with *S. epidermidis* constant activity at 4°C and 25°C with constant loss at 50°C. P_8 activity against *E. coli* was increased at 4°C from zero to 6.0 ± 0 then 7.0 ± 0 at 25°C then 1.0 ± 0 at 50°C and lost at 70°C. In case of *S. typhi*, the pattern of P_8 activity is different than any other microbe used. The activity increased from zero and reached maximum at 50°C then lost at 70°C. While an organic molecule, the control chloramphenicol, showed consistency in its activity at different temperature.

These findings support the view that specific conformation of peptide is needed for its function. Any change in conformation based on temperature leads to the loss of function i.e., antimicrobial activity. The study of Ngai and Ng (2004) also pointed out the functional activity of polypeptide isolated from Chinese white cabbage was stable between 10°C and 40°C.

Effect of different pH: The effect of pH on the antimicrobial activity of purified peptides of seed (S_4, S_5) and pod (P_7, P_8) was checked against *S. aureus, S. epidermidis, E. coli* and *S. typhi* because purified peptides have shown effectiveness against these microbes. The results obtained are shown in Table 4. With pH change, the antimicrobial activity of peptides also got changed. At pH 4 and 8, all peptides became inactive against all the microbes tested. Peptide S_4 showed gradual increase in activity against *S. aureus* and *E. coli,* the highest activity was found at pH 7. Both S_4 and S_5 have shown constant activity at pH 5 -7 against *S. epidermidis* and *S. aureus*, though *S. aureus* is more sensitive to S_5 than *S. epidermidis.* Both S_4 and S_5 showed similar pattern against *S. typhi* with highest at pH 6 while S_5 also showed highest activity against *E. coli* at pH 7. Correspondingly, in case of pod peptides, both P_7 and P_8 showed zero activity and loss of activity at pH 4 and pH 8 except P_8 against *E. coli* which showed no change in sensitivity at all pH used. P_7 showed maximum activity against *S. aureus* at pH 6-7, against *S. typhi* at pH 6 while against *S. aureus* and *S. typhi* P_8 showed increase in activity at pH 5 reaching maximum at pH 6 and 7. In case of *S. epidermid* low activity was noted at pH 5-6. In contrast to effect of pH on activity of purified peptide, control sample i.e. chloramphenicol showed consistency in its activity.

Table. 4. Effect of pH on the antimicrobial activity of purified peptides of seed (S_4, S_5) and pod (P_7, P_8) against various bacteria. Values are the mean ± SEM of triplicate determination from a representative experiment of the three experiments performed. Cam [34] Chloramphenicol (34 mg / ml).

pH	S_4 (6µg/10µl protein conc.)				S_5 (4.35µg/10µl protein conc.)			
	S. aureus	*S. epidermidis*	*E. coli*	*S. typhi*	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhi*
4	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
5	1 ± 0.21	2.0 ± 0.0	3 ± 0.0	6 ± 0.06	13 ± 0.1	-	4 ± 0.0	6.0 ± 0.17
6	5 ± 0.21	2.0 ± 0.17	4 ± 0.17	11 ± 0.2	13 ± 0	-	4 ± 0.1	11.0 ± 0.21
7	14 ± 0	2.0 ± 0.06	6 ± 0.0	10 ± 0.2	13 ± 0.1	-	5 ± 0.2	9.0 ± 0.0
8	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Cam [34]	24.0	16.0	17.0	19.0	24.0	-	17.0	19.0
	P_7 (0.9µg/10µl protein conc.)				P_8 (1.2µg/10µl protein conc.)			
4	0.0	-	-	0.0	0.0	0.0	4 ± 0.21	0.0
5	0.0	-	-	5 ± 0	13 ± 0.06	1 ± 0.0	4 ± 0.17	5 ± 0.0
6	14 ± 0	-	-	11± 0.21	14 ± 0	1 ± 0.06	4 ± 0.06	10 ± 0.2
7	14 ± 0	-	-	9± 0	14 ± 0	0.0	4 ± 0.06	10 ± 0.1
8	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Cam [34]	24.0	16.0	17.0	19.0	24.0	-	17.0	19.0

These results show that activity of purified peptides are pH-dependent and varies for different microbes. The peptides are active between pH 5-7 in most of the cases and these become inactive at pH 4 and pH 8 i.e. at more acidic and more basic ranges. These results of purified peptides are not in accordance with the broad range antimicrobial substances which have high antimicrobial activity in pH range of 2.0-9.0 as studied by Risoen *et al.*, (2004). Similarly the stability of polypeptide isolated from Chinese white cabbage retained between pH 4-11 as described by Ngai & Ng (2004). The results of our study revealed that purified peptides are stable between acidic to neutral ranges of pH.

Effect of different enzymes: Purified peptides of seed (S_4, S_5) and pod (P_7, P_8) were further checked for their stability in antimicrobial activity against *S. aureus, S. epidermidis, S. typhi* and *E. coli* because purified peptides have shown effectiveness against these microbes. After treating the purified peptides with proteolytic enzymes i.e., proteanase

K and trypsin, all peptides became inactive providing the evidence that proteolytic enzymes have degraded the peptides which led to the loss of antimicrobial activity (Table 5). These results are comparable to the broad range antimicrobial substances which were sensitive to proteanase K (Risoen *et al.*, 2004).

Effect of various concentrations: Various bacteria against which purified peptides obtained from seed and pod showed significant antimicrobial activity were subjected for the determination of minimal inhibitory concentration (MIC) using the various concentrations of antimicrobial peptide preparation i.e., 0-200µg / ml on the apparent proliferation of these organisms. Results are given in Fig. 5 indicating MIC of seed peptides (S_4, S_5) and pod peptides (P_7, P_8) against *S. aureus, S. epidermidis, S. typhi* and *E. coli*. Our findings show that *S. aureus* was the most sensitive microbe with the lowest MIC value of 75 µg / ml when treated with seed peptides i.e., S_4 and S_5 while both pod peptides i.e., P_7 and P_8 showed MIC of 100 µg /ml. MIC value of 75µg / ml and 100µg /ml is comparable to 72µg /ml and 108µg /ml MIC value, respectively of Hf–1 peptide isolated from housefly larvae (Hou *et al.*, 2007). MIC value was considered as 40 - 60 % decrease in apparent proliferation of test culture. Therefore antimicrobial effect of isolated peptides was found to be concentration dependent. High MIC values of *S. epidermidis, S. typhi* and *E. coli* are indicative that either the isolated peptides are less effective on some gram positive bacteria or that the organisms have the potential of developing antibiotic resistance while the low MIC value against *S. aureus* is an indication of the efficacy of the isolated peptides.

Table. 5. Effect of enzymes on the antimicrobial activity of purified peptides of seed (S_4, S_5) and pod (P_7, P_8) against various bacteria. Values are the mean ± SEM of triplicate determination from a representative experiment of the three experiments performed. Cam [34] Chloramphenicol (34 mg/ml).

Enzymes	S_4 (6 µg/10 µl protein conc.)				S_5 (4.5 µg/ 10 µl protein conc.)			
	S. aureus	*S. epidermidis*	*E. coli*	*S. typhi*	*S. aureus*	*S. epidermidis*	*E. coli*	*S. typhi*
Proteinase K	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Trypsin	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Cam [34]	24.0	16.0	17.0	19.0	24.0	-	17.0	19.0
	P_7 (0.9 µg/ 10µl protein conc.)				P_8 (1.2µg/10µl protein conc.)			
Proteinase K	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Trypsin	0.0	0.0	0.0	0.0	0.0	-	0.0	0.0
Cam [34]	24.0	16.0	17.0	19.0	24.0	-	17.0	19.0

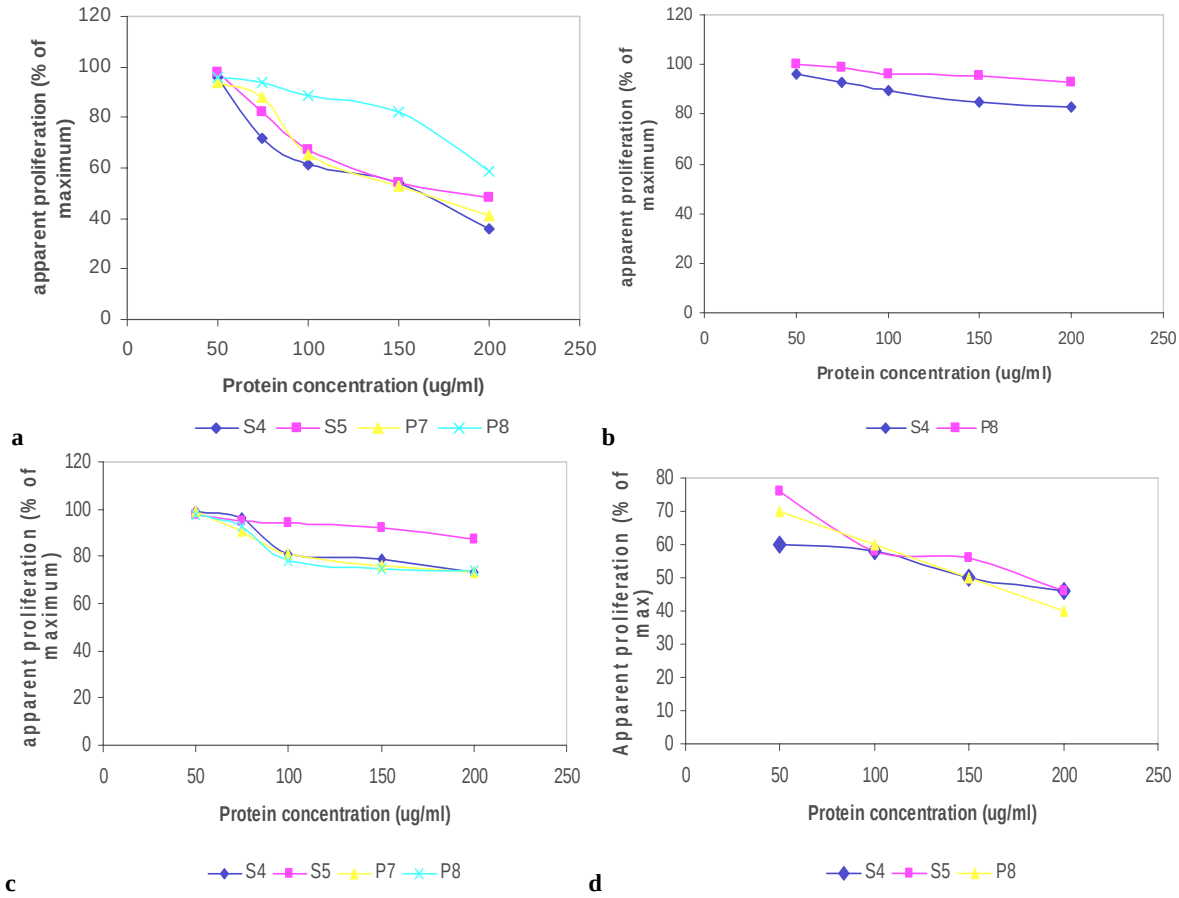


Fig. 5. Effect of various concentrations of isolated peptides (S_4, S_5 and P_7, P_8) on the apparent proliferation of a: *S. aureus*,b: *S. epidermidis*, c: *S. typhi*, d: *E. coli.*

Conclusions

Isolation and characterization of potential peptides from *Pisum sativum* L. and the demonstration of broad spectrum of antibacterial activity by these purified peptides against a number of bacteria can assist discovery of novel antibiotic substances that could serve as selective agents against infectious diseases, chemotherapy as well as their control. This study has also unlocked the possibility of the use of this plant, especially the non-edible part of peas which goes as waste, for the development of drug for human consumption possibly for the treatment of gastrointestinal, urinary tract, wound infections and typhoid fever.

References

Anonymous. 2008. World Health Organization. Traditional Medicine. Fact sheet. www.who.int/mediacentre/factsheets/fs134/en/

Bauer, R.W., M.D.K. Kirby, J.C. Sherris and M. Turck. 1966. Antibiotic susceptibility testing by standard single disc diffusion method. *Amr. J. Clin. Pathol*., 45: 493-496.

Borchardt, J.R., D.L. Wyse, C.C. Sheaffer, K.L. Kauppi, R.G. Fulcher, N.J. Ehlke, D.D. Biesboer and R.F. Bey. 2008. Antioxidant and antimicrobial activity of seed from plants of the Mississippi river basin. *J. Med Plants Res*., 2(4): 081-093.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *J. Anal Biochem*., 72: 248-254.

Carol, J.C., C. Song, G. LaMonte, K. Fetalvero, K. Hinchman, H. Phan, M. Pineda, K. Robinson and G.P. Schneider. 2005. Identification and partial characterization of the enzyme of *omega*: one of five putative DPP IV genes in *Drosophila melanogaster*. *J. Insect Sci*., 5: 26.

Davies, D.R., G.J. Berry, M.C. Heath and T.C.K. Dawkins. 1985. Pea (*Pisum sativum* L.). In: Grain Legume Crops (Eds.): R.J. Summerfield and E.H. Roberts. Williams Collins Sons and Co. Ltd, London, UK: 147-198.

Feng, J., F. Yuani, Y. Gao, C. Liang, J. Xu, C. Zhang and L. He. 2003. A novel antimicrobial protein isolated from potato (*Solanum tuberosum*) shares homology with an acid phosphatase. *J. Biochem*., 376(2): 481-487.

Hou, L., S.Y. Shi, P. Zhai and G.Le. 2007. Inhibition of food-borne pathogens by Hf-1, a novel antibacterial peptide from the larvae of the housefly (*Musca domestica*) in medium and orange juice. *J. Food Control*., 18(11): 1350-1357.

http://www.mindbranch.com, Frost & Sullivan. 2004. Strategic analysis of the therapeutic peptide market in Europe industry research. Online article.html.

Hu, S.H., J.C. Wang, H.F. Kung, J.T. Wang, W.L. Lee and Y.H. Yang. 2004. Antimicrobial effect of extracts of cruciferous vegetables. *Kaohsiung J. Med Sci*., 20(12): 591-599.

Laemmli, U.K. I970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *J. Nature*, 227: 680-685.

Marcos, J.F., A. Muñoz, E. Pérez-Payá, S. Misra and B. López-García. 2008. Identification and rational design of novel antimicrobial peptides for plant protection. *J. Annu Rev Phytopathol*., 46: 273-301.

Mariângela, S., S. Diz, A.O. Carvalho, R. Rodrigues, A.G.C. Neves-Ferreira, M.D. Cunha, E.W. Alves, A.L. Okorokova-Façanha, M.A. Oliveira, J.Perales, O.L.T. Machado and V.M. Gomes. 2006. Antimicrobial peptides from chilli pepper seeds causes yeast plasma membrane permeabilization and inhibits the acidification of the medium by yeast cells. *Biochem. et Biophys Acta*. 1760(9): 1323-1332.

Marx, V. 2005. Watching peptide drugs grow up. *J. Chem and Eng. News*, 83(11): 17-24.

Muehlbauer, F.J. and A. Tullu. 1998. New CROP Fact SHEET, *Pisum sativum* L. http://www.hort.purdue.edu/newcrop/cropfactsheet/ grass- pea.html

Ngai, P.H. and T.B. Ng. 2004. A napin-like polypeptide from dwarf Chinese white cabbage seeds with translation-inhibitory, trypsin-inhibitory, and antibacterial activities. *J. Peptides*., 25(2): 171-6.

Ngai, P.H., Z. Zhao and T.B. Ng. 2005. Agrocybin, an antifungal peptide from the edible mushroom *Agrocybe cylindracea*. *J. Peptides*., 26(2): 191-6.

Oard, S., M.C. Rush and J.H. Oard. 2004. Characterization of antimicrobial peptides against a US strain of the rice pathogen *Rhizoctonia solani*. *J. Appl. Microbiol*., 97(1): 169-80.

Risoen, P.A., P. Ronning, I.K. Hegna and A.B. Kolsto. 2004. Characterization of a broad spectrum range antimicrobial substances from *Bacillus cereus*. *J. App. Microbiol*., 96(4): 648-655.

Ruissen, A.L.A., J. Groeninck, E.J. Helmerhorst, E. Walgreen-Weterings, W. Van't Hof, E.C.I. Veerman and A.V. Nieuw-Amerongen. 2001. Effects of Histatin 5 and derived peptides on *Candida albicans*. *J. Biochem*., 356(2): 361-368.

Talas, T. 2004. Screening antimicrobial activities of basic protein fractions from dry and germinated wheat seeds. *J. Biologia Plantarum*, 48(4): 583-588.

Wang, F. 2003. Effect of antimicrobial proteins from porcine leukocytes on *Staphylococcus aureus* and *Escherichia coli* in comminuted meats. *J. Meat Science*., 65: 615-621.

Wright, G.D. 2000. Resisting resistance: new chemical strategies for battling superbugs. *J. Chem and Bio*., 7(6): 127-132.

Xia, L. and T.B. Ng. 2005. Isolation of alliumin, a novel protein with antimicrobial and antiproliferative activities from multiple-cloved garlic bulbs. *J. Peptide.*, 26(2): 177-183.

(Received for publication 28 April 2010)