Antimicrobial and cell surface hydrophobicity effects of chemically synthesized fermented foxtail millet meal fraction peptide (FFMp10) mutants on *Escherichia coli* ATCC 8099 strain

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Abstract

This study aimed to verify the effect of chemically synthesized short peptides (LHALLL), previously identified from a fermented foxtail millet meal fraction (FFMp10) on the cell surface hydrophobicity (CSH), biomass growth, DNA binding ability of *Escherichia coli* ATCC 8099. Proteolytic responses of these peptides were also tested using the high performance liquid chromatography (HPLC). The HPLC elution profiles revealed that FFMp10 and its four mutants (Mp1, Mp2, Mp3 and Mp4) peptides responded to 1 h pepsin treatment. However, FFMp10 and Mp1 showed reduction in the retention time, and Mp2 increased the retention time, though Mp3 and Mp4 were more resistant to this proteolytic digestion than FFMp10 and Mp1. Values of CSH were significantly higher (P<0.05) with FFMp10, Mp1 and Mp2 2 h treatment on bacteria, whereas, lowered with Mp3 and Mp4 treatment. Overall peptide treatment which lowered the rate of bacterial biomass growth when compared to control. Slight *E. coli* ATCC 8099 DNA bindings were observed in lane 2, 3 and lane 3 respectively for Mp1 and Mp2 incubation. This suggests that these short peptides reduced hydrophobicity of bacterial, but not the viability. Thus, provide evidence of effect of these short peptides on the *E coli* ATCC 8099 growth.

Introduction

Antimicrobial peptide with a short (4-7 amino acid residues) size and a simple amino acid composition would be a more favorable lead molecule to reduce production costs and to facilitate pharmaceutical optimization (Nan *et al.*, 2012). In this line, potent antimicrobials have been produced based on natural peptides and the active domains of larger antimicrobial proteins (Ntwas*a et al*., 2012). Short, positively charged antimicrobial peptides (AMPs), have been found in plants and animals as part of the innate immune system that helps to defend against invading microorganisms (Huo *et al*., 2011). Hydrophobic amino acid stretches can be used to enhance bactericidal potency of ultra-short AMPs with limited toxicity (Pasupuleti *et al.*, 2009). Enzymatic instability of a peptide could limit its in vivo use. Studies indicated that in the gastrointestinal

tract, amino acid peptides are more readily absorbed than the amino acid themselves (Kodera *et al.*, 2006; Gilbert *et al*., 2008). Digestive enzymes such as pepsin, trypsin are suggested to exert a synergistic effect on protein digestion in the body (Getz *et al.*, 2011; Dickey and Potter 2011).

Plants, animals and microorganisms commonly use the release of antimicrobial peptides as a defensive strategy against pathogenic microorganism and stress (Ntwasa *et al*., 2012; Kamara *et al*., 2012). It is generally believed that a protein resist proteolytic digestion in the digestive tract, retains sufficient structural integrity and that increases the probability of stimulating natural defense of the organism against invading pathogens (Ntwasa *et al*., 2012; Guani-Guerra *et al*., 2010; Hannan *et al.*, 2014). Detecting foodborne pathogenic bacteria such as *Escherichia coli* is an important factor ensuring food safety (Liu 2015; Wang *et al*., 2015; Gregory

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Table 1. Estimated physiochemical properties of identified Leu rich peptide (FFMp10 fraction)
from fermented foxtail millet and its replicates (Mp1, Mp2, Mp3 and Mp4 peptides)

Peptides	Molecular weights (Da)	Hydrophobicity contributions**	Isoelectric points*	Net charges at neutral pH*	Sequences
FFMp10	678.88 g/moL	2.62	pH 7.85	0	LHALLL
Mp1	697.92 g/moL	2.14	pH 11.04	1	LRALLL
Mp2	735.97 g/moL	3.1	pH 10.1	1	LKALLL
Mp3	721.9 g/moL	1.03	pH 11.04	1	RHALLL
Mp4	721.9 g/moL	1.03	pH 11.04	1	LHALLR

* Innovagen's peptide property calculator *(http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp)*; ** Hydrophobicity base on amino acids contributions (partition coefficient log P).

and Mello, 2005). *E. coli* is commonly found in the gut of humans and warm-blooded animals. Findings from structural activity analysis of AMPs revealed that the cell penetrating efficiency and affinity to DNA are critical factors in determining the potency of antimicrobial peptides; and binding of ultra-short peptides to bacterial DNA or DNA fragments could be a mechanism to show the antimicrobial efficacy of these compounds (Huo *et al.*, 2011; Hao *et al.*, 2013).

Hydrophobicity appears to be imparted by different chemical components of the cell wall in different bacteria; these components include lipoteichoic acids and proteins (Thwaite *et al.*, 2009; Van der Mei and Busscher 2012). The cell surface hydrophobicity (CSH) of bacteria is been recognized as a physical measurable macroscopic characteristic which, reflects the ratio of hydrophobic to hydrophilic cell envelope constituents (Jones *et al.*, 1996). CSH responds to a wide variety of environmental factors and, it's suggested to be involved in several activities like cell-to-cell interaction, adherence of bacteria to solid surfaces and host tissue, partitioning at liquid–liquid, solid–liquid or liquid–air interfaces, resistance of cells to specific treatments like organic solvents or antibiotics, which activities are essential in technologies and natural processes (Jones *et al.*, 1996; Choi *et al.*, 2013; Zikmanis *et al.*, 2007).

A characterized and identified FFMp10 peptide fraction from foxtail meal fermentation using *Lactobacillus paracasei* Fn032 was suggested to inhibit the growth of *E. coli* ATCC 8099 (Amadou *et al.*, 2013; Amadou *et al.*, 2013); however there is little information about the effect of FFMp10 peptide on the cell surface hydrophobicity, DNA binding ability of *E. coli* ATCC 8099 and its proteolytic response. Therefore, we set to assess the effect of chemically synthesized FFMp10 peptide replicate residues on

the cell surface hydrophobicity, biomass growth and DNA retardation ability of E. coli ATCC 8099. Furthermore, these peptides responses to pepsin proteolysis were also tested.

Materials and Methods

Materials

The chemically synthesized peptides (Mp1, Mp2, Mp3 and Mp4 are replicate of FFMp10) with 99% purity tested by the company were purchased from Shanghai Biotech Bioscience & Technology Co., Ltd (Shanghai, China). All other chemicals and reagents (from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were of analytical grade.

Proteolytic digestion

Digestion of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides by pepsin using 0.2 µg/mL pepsin in PBS and 50 µg/mL peptide followed by incubation at 37oC for 1 h were carried out. The progress of peptides cleavage were monitored chromatographically using a HPLC (Shimadzu LC-20A, Japan).

Calculation of hydrophobicity contributions

The hydrophobicity contributions (Table 1) were calculated based on amino acids contributions (partition coefficient log P); which was based on the references given by Gao et al. (1995) as hydrophobic contribution constants of amino acid residues to the hydrophobicities of peptide.

Determination of bacterial cell surface hydrophobicity

Bacteria (*E. coli* ATCC 8099) were maintained as glycerol stocks and stored at -70°C. Stock cultures of all microorganisms were grown in nutrient agar broth

at 37ºC for 18 h. Aliquots of single bacteria colonies were transferred into a test tube containing sterilized distilled water, centrifuged at 10000 rpm for 1 min using an Eppendorf centrifuge 5430 (Hamburg, Germany) and the deposited bacterial cells pellets were washed three times. The *E. coli* ATCC 8099 (OD600= 0.4 ± 0.02) pellets were sampled into 2 parts of which one part was directly re-suspended in sterile phosphate buffered saline (0.1 mM PBS, pH 7.2) and the other part was treated with FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides (1 mg/mL) for 2 h prior to bacterial cell surface hydrophobicity (CSH) assay as described by Jones *et al.* (1996) with little modification testing the bacterial adherence to hydrocarbons (BATH).

After 2 h of incubation the *E-coli* ATCC 8099 cells were washed twice with sterilized PBS and then re-suspended in PBS. Then 2.4 mL of bacterial-sterilized PBS suspension were added to 0.2 mL xylene and vortex mixed at constant speed for 3 min. After phase separation, the lower aqueous layer was carefully removed and the absorbance was measured at 600 nm (SPM-UV2300 spectrophotometer, Shanghai, China). The hydrophobicity was expressed as a percentage of the applied cell suspension absorbance which had been excluded from the aqueous phase. The 2 h treated *E-coli* ATCC 8099 cells with peptides and cells suspended in sterilized PBS were then grown in sterilized liquid broth Mann Rogosa Sharpe (MRS) to test their biomass growth at different times (4, 8, 12, 18 and 24 h).

DNA binding assay

Gel-retardation experiments were performed using 2 µL of *E. coli* ATCC 8099 genomic DNA (100 ng) for each reaction, 0.8 µL each of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide concentrations (0.5, 0.8 and 1.0 mg/mL) were mixed with 2 µL of binding buffer (10% Ficoll 400, 10 mM Tris-HCl; pH 7.5, 50 mM EDTA, 0.25% bromophenol blue). The reaction mixtures were incubated at room temperature for 10 min and loaded to 0.8% agarose gel with 2 µg/mL ethidium bromide in 0.5× Tris borate-EDTA buffer then subjected to electrophoresis (Zhang *et al.*, 1999).

Statistical analysis

Analysis of variance (ANOVA) was performed on bacterial cell hydrophobicity data, and significant differences in mean values were evaluated at 95% confidence test, using SPSS version 19.0 (SPSS, Chicago, IL, USA).

Results and Discussion

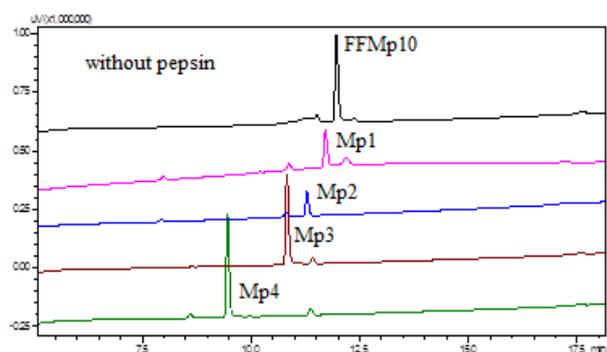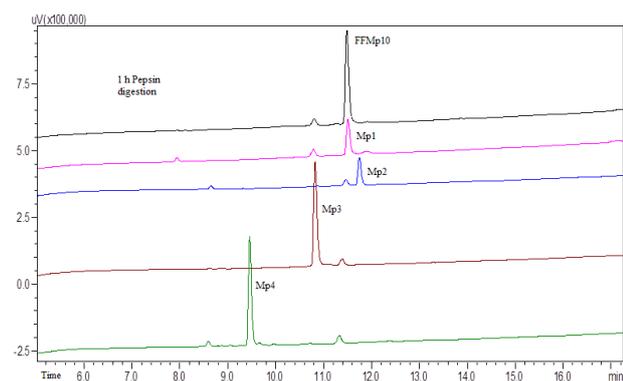*Estimated physicochemical properties of the peptides*

The Arg and Lys residues within a given peptide are known for their role in DNA binding (Deber *et al.*, 2001; Huo *et al.*, 2011; De Rouchey *et al.*, 2013). The theoretical estimated physiochemical properties of identified Leu rich peptide (FFMp10 fraction) from fermented foxtail millet meal and its replicates (modified peptides: Mp1, Mp2, Mp3 and Mp4) are presented in Table 1. In our previous report we demonstrated that the Leu rich peptide had significant contribution to the high hydrophobicity of peptide sequences (Amadou *et al.*, 2013). Substitution of His by Lys at second amino acid sharply increased the hydrophobicity contributions value of Mp2 to 3.1, higher than its counterparts. Moreover, substitution of Arg and Lys in the sequence FFMp10 increased both the net charge at neutral pH and respective isoelectric points of the replicate peptides (Table 1). The net positive charge and the hydrophobicity are critical factors in determining the antimicrobial potency of the peptides (Zikmanis *et al.*, 2007; Rosenfeld *et al.*, 2010; Sousa *et al.*, 2009).

Proteolytic digestion

The susceptibilities of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides to pepsin proteolysis HPLC elution profiles are shown in Figure 1. Previously, we have demonstrated the HPLC elution profiles of FFMp10 sequence proteolysis against trypsin (Amadou *et al.*, 2013). The HPLC elution profiles of FFMp10 and its four replicates (Mp1, Mp2, Mp3 and Mp4) peptides showed some response to 1 h pepsin treatment (Figure 1). However, the FFMp10 and Mp1 revealed some reduction in the retention time, and Mp2 appeared to add in retention time, whereas Mp3 and Mp4 were more resistant to this proteolytic digestion than FFMp10 and Mp1 indicates that locations besides the type of amino acid also play a vital role in peptide digestion. Previous studies demonstrated that structural dimension is an important factor for peptides in their susceptibilities to proteolytic degradation (Getz *et al.,* 2011; Bracci *et al.*, 2003; De Zotti *et al.*, 2009). Interestingly, both Arg and Lys residues contributed remarkably to the proteolytic activities of these synthetized peptides, thus, this may extend interest in their bioactivities.

Changes in bacterial cell hydrophobicity

Majority of vital activities in Gram-negative bacteria such as sensory, protective, transport and energy generation functions are dependent on the structural and functional properties of the cell envelope containing the cytoplasmic and the outer membrane as the principal components (Nikaido,

Peptides	Without pepsin		Pepsin treatment	
	Retention time (min)	Area	Retention time (min)	Area
FFMp10	11.963	2043917	11.485	1869462
Mp1	11.711	956925	11.507	732584
Mp2	11.284	515704	11.746	502100
Mp3	10.824	2010811	10.823	2011080
Mp4	9.459	2005355	9.458	1893683

Figure 1. HPLC elution profiles (retention times and areas) of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides sequences treated with pepsin. Experimental conditions: Galaksil EF-C18 (H), 5μm 4.6*250 reverse-phase column; gradient from 2 to 90% B in 25 min A = 0.1% TFA in a 95:5 (v/v) mixture of H_2O/CH_3CN; B = 0.1% TFA in a 5:95 (v/v) mixture of H_2O/CH_3CN]. The absorption was monitored at 220 nm
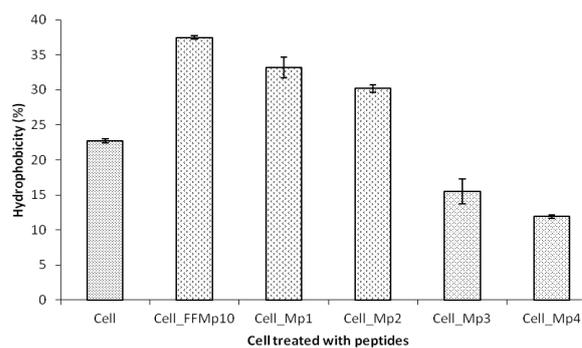

Figure 2. Changes in the *E. coli* ATCC 8099 cell surface hydrophobicity by FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide treatment
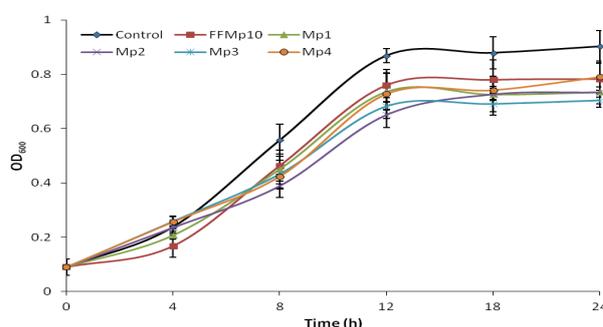

Figure 3. Biomass of *E. coli* ATCC 8099 growth following FFMp10, Mp1, Mp2, Mp3 and Mp4 peptide treatment

2003). *E coli* ATCC 8099 cells treated with FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides showed significant ($P < 0.05$) changes in cell surface hydrophobicity values (Figure 5.2). The involvement of net positive charge and the hydrophobicity indexes (Table 5.1) showed that FFMp10 had higher impact on the CSH followed by Mp1 and Mp2; whereas Mp3 and Mp4 CSH values were considerably lower when compared to the E coli ATCC 8099 cells that were not treated with peptides. Peptides with higher hydrophobicity index contributed to higher CSH percentages (Figure 5.2). Indeed, more profound increases of CSH values were observed after the FFMp10, Mp1 and Mp2

treatment. Study suggested that high CSH values tend to promote the self-aggregation of bacteria and it enhances the possibility of modifying cell surface properties (Zikmanis *et al.*, 2007). The hydrophobicity of the cell surface enhances in vivo bacteria pathogenicity by either increasing the rate of phagocytosis or enhancing the colonization of mucosal surfaces (Thwaite *et al.,* 2009), in this line, Mp3 and Mp4 could provide prominent action against the *E coli* ATCC 8099 activity. Substitutions of Arg at end terminals of Mp3 and Mp4 might have contributed to this activity by reducing the CSH of this strain.

Previous study has shown that hydrophobic peptides can cause alterations to the surface morphology of bacterial cells (Tagai *et al.*, 2011). The present study demonstrated that disparity in amino acid composition and sequence of FFMp10, Mp1 and Mp2 induced higher CSH in *E. coli* ATCC 8099 cells compared to Mp3 and Mp4 with lesser CSH display, and that might have resulted in morphological changes. The Gly/Leu-rich antimicrobial peptides with ability to inhibit the growth of Gram-negative bacteria such as *E. coli* were reported (Sousa *et al.*, 2009); similarly, this study suggests that the
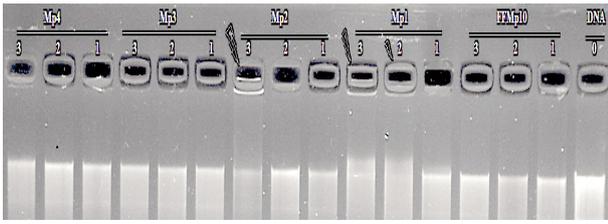
Figure 4. Interaction of *E. coli* ATCC 8099 genomic DNA with peptides; (2 µL of 100 ng DNA) was incubated with 8 µL (0.5, 0.8 and 1.0 mg/mL, lanes 1, 2 and 3 respectively) of FFMp10, Mp1, Mp2, Mp3 and Mp4 peptides at room temperature for 10 min. The reaction mixtures mixed with binding buffer (2 µL) were applied to a 0.8% agarose gel electrophoresis

synthesized short peptides replicate (6 residues rich in Leu) from FFMp10 fraction induced changes (Figure 5.2) across the *E coli* ATCC 8099 cell membrane in a similar trend to the Gly/Leu-rich peptides.

Bacterial biomass growth

Antimicrobials, for instance, first have to approach an organism and interact with its cell surface before they can become effective. Bacteria have a natural tendency to adhere to surfaces as a survival mechanism, they can adapt quickly to a new environment triggered by environmental signals to change their phenotypic appearance, but it is of apparent advantage that not all clones in a population follow the same trend (Choi *et al.*, 2013).

When a microorganism is introduced into the fresh medium, it takes some times to adjust with the new environment; therefore, the effect of these peptides (FFMp10, Mp1, Mp2, Mp3 and Mp4) treatment on *E coli* ATCC 8099 followed the same trend. Figure 5.3 shows the exponential or Logarithmic (log) phase of bacterial biomass growth after adaptation time up to 12 h, followed by stationary phase, that all the treated cells have lower growth rate than the control. The survival mechanism by this strain demonstrated that the peptides must have played an important role on the starvation of E coli ATCC 8099. Interestingly, FFMp10 with lower net charges at neutral pH exercised more effect on the cell growth retardation and also resulted later in the highest CSH formation (Table 5.1, Figure 5.2). Thus, this could have resulted into the more biofilm formation which helps strain in its adaptation to the new environmental conditions (Van der Mei and Busscher, 2012; Choi *et al.*, 2013).

DNA binding

The CSH of *E. coli* ATCC 8099 appears to be imparted by the effect of the synthetized peptides (Figure 5.2); however, efficiency and the DNA binding ability were critical factors for determining the antimicrobial potency of a peptide (Huo *et al.*, 2011; Hao *et al.*, 2013; DeRouchey *et al.*, 2013; Ulvatne *et al.*, 2004). Therefore, the DNA binding ability of FFMp10, Mp1, Mp2, Mp3 and Mp4 were evaluated using an electrophoresis gel mobility assay (Figure 5.4). The positively charged peptides have ability to bind to phosphate negatively group in the DNA back bone (Deber *et al.*, 2001). The migration of DNA was not retarded in most cases even with increased peptide concentrations. However, a slight attempt to retard DNA migration was observed in Mp1 and Mp2 to DNA ratio in their lanes 2, 3 and lane 3 respectively (Figure 5.4). There was significant E. coli ATCC 8099 DNA binding affinity which was similar to the trend observed by Huo *et al.* (2011) that demonstrated that MUC7 12-mer a 12 amino acid peptide did not inhibit DNA electrophoretic mobility at a mass ratio of 500:1. Thus, this suggests that the peptide may have antimicrobial activity (Amadou *et al.*, 2013) but with no ability to retard bacterial DNA migration. Furthermore, the antimicrobial activity corresponded with the CSH data showed that the *E. coli* ATCC 8099 cell wall surface was affected by changes in surface hydrophobicity instead of the bacteria intracellular DNA binding. This was in agreement with our previous study where we demonstrated that short peptides of such kinds have difficulties in retarding DNA migration (data not shown). In contrary to a study by Ulvatne et al. (2004) who demonstrated that high concentration of lactoferrin at mucosal surface and in milk did not bind bacterial DNA.

Conclusion

In this study, we demonstrated that the synthesized Leu rich short peptides displayed some proteolytic resistance to pepsin proteolysis as a result of Arg and Lys residues substitutions in the FFMp10 peptide sequence. The observed multiple relationships between the CSH values and theoretical hydrophobicity indexes to overall biomass growth and DNA binding of *E coli* ATCC 8099 strain were attained. On the basis of these data, short peptides reduced hydrophobicity, and slightly the viability of *E coli* ATCC 8099; moreover, the Mp3 and Mp4 peptides may provide an attractive function against *E coli* ATCC 8099 CSH activity. Further studies to understand these peptides involvement in in vivo activity may be helpful in elucidating the development of enzyme resistant stable short peptide ligands suitable for possible applications.

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