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Potential MIC of Bioactive Peptides from Fermented Bovine Milk to Inhibit Bacterial Pathogens

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A B S T R A C T

Bovine milk proteins have been identified that possess broad spectrum antimicrobial activity. *In vitro*, these proteins upon degradation by digestive enzymes or microbial enzymes have been shown to release antimicrobial peptides (AMPs), which exhibit unique mechanism for killing bacteria compared with current antibiotics. These AMPs selectively binds to the outer lipid membrane of the bacterium and form blisters and pores, which eventually result in lyses of the cell leading to cellular death. Present research has been carried out to find out the minimal inhibitory effect of peptide released during fermentation under controlled condition on *Staphylococcus aureus* and *Escherichia coli*. Bioactive peptides generated during fermentation were separated using different techniques and tested for antimicrobial activity. These peptides first purified with gel filtration technique and then subjected to reverse phase high performance liquid chromatography. Broth dilution and well diffusion methods were used to determine the lowest concentration of antimicrobial peptides agent (minimal inhibitory concentration, MIC) that can inhibit the growth of selected pathogen; we find that under defined test conditions, AMPs inhibits the visible growth of the bacterium being investigated. This study demonstrated that bioactive peptides having antimicrobial acitivity generated during fermentation with *L. helveticus* showed a minimum of 8mm zone of inhibition against gram positive *S. aureus* and 9mm against gram negative *E. coli* on plates containing 20µl peptide concentration as compared to respective plates or broth tubes which determines the lowest concentration of the assayed antimicrobial peptide, minimal inhibitory concentration, (MIC). The amount of peptide as calculated for 20µl peptide suspension was 0.34 mg/ml. Therefore we can conclude that 0.34 mg/ml of may be considered as minimal inhibitory concentration for the inhibition of growth of *S. aureus* and *E. coli.* Further study and animal trials must be carried out to decide final dose of such peptide to be used for therapeutic agent or as in food preservation.

Introduction

Bioactive peptides are defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen and Pihlanto, 2006). The beneficial effects are attributed to different properties found in peptides such as

antimicrobial (Rajanbabu and Chen, 2011; Bhagat Singh *et al.*, 2012), antioxidant (Bhagat Singh and Rattan Chand, 2006; Sarmadi and Ismail, 2010), antithrombotic (Wang and Ng, 1999), anti-hypertensive (Erdmann, 2008) and immunomodulatory activities (Gauthier *et al*., 2006), among others. Food safety is a growing concern of great importance worldwide. Recently, the estimated costs of diseases caused by food borne pathogens was about $152 billion in the United States (Scharff, 2010), The consumption of processed foods with chemical preservatives has led to increased consumer concern and the demand for more natural and minimally processed foods. As a result, researchers have shown a growing interest in natural antimicrobial agents such as certain peptides. The value of proteins as an essential source of amino acids is well documented, but recently it has been recognized that dietary proteins exert many other functionalities in vivo by means of biologically active peptides (Agyei, 2011) A number of bioactive peptides have been identified in milk proteins, such as casein and whey proteins, where they are present in an encrypted form, stored as propeptides or mature C-terminal peptides that are only released upon proteolysis (Gobbetti *et al*., 2002; Kamysu *et al*., 2003).

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to an antimicrobial agent and also to monitor the activity of new antimicrobial agents. MIC can be helpful in establishing the level of resistance of a particular bacterial strain and can substantially affect the decision to use certain antimicrobial agents. Minimum Inhibitory Concentration (MIC) test is a well-established assay for the biostatic (growth-inhibiting) activity of liquid antimicrobials. It is rapid, relatively inexpensive, and reliable. The MIC method measures the effect of decreasing concentrations of antiseptic over a defined period of time in terms of inhibition of microbial population growth. The concentration of drug required to produce the effect is defined as the Minimum Inhibitory Concentration and is normally several hundred to thousands of times less than the concentration found in the finished dosage form.

Minimum Inhibitory Concentration (MIC) testing define a test material's potency in terms of the concentration at which it will inhibit growth of (Minimum Inhibitory Concentration, or MIC) or completely kill (Minimum Bactericidal Concentration, or MBC) 1 x 10^6 (one million) challenge microorganisms during a 18 to 20 hour period of incubated (35 ± 2°C) exposure. Currently, there are a few web-based, freely accessible MIC databases. Clinicians use MIC scores to choose which antibiotics to administer to patients with specific infections and to identify an effective dose of antibiotic. This is important because populations of bacteria exposed to an insufficient concentration of a particular drug or to a broad-spectrum antibiotic can evolve resistance to these drugs. Therefore, MIC scores aid in improving outcomes for patients and preventing evolution of drug-resistant microbial strains. The MIC test can be done on a very small scale without using too much antimicrobial agent. This is important for experimental antimicrobials such as biologically synthesized antimicrobial peptides (Bhagat Singh and Renu Singh., 2011).

Materials and Methods

Preparation of Peptide Stock solution

Activation of Culture

Activation of *Lactobacillus helveticus* NCDC 292, culture was done using MRS broth which is specific for Lactic Acid Bacteria. Five ml of MRS broth was taken in 15 ml test tube in duplicate. After sterilization, it was cooled at room temperature. The lyophilized ampoule of *L. helveticus* was broken aseptically in laminar air flow and small amount (one loop full) of the dried culture was transferred into tube containing MRS broth. It was mixed properly using vortex shaker and then incubated at 37°C in an incubator for 24 to 48 hours. After 48 hours the tubes were observed for the growth and purity of culture was tested.

Fermentation of milk

Bovine milk was sterilized 250ml in each flask, the milk was then cooled up to room temperature and then flask were inoculated with all three @ 4.0% and incubated at 37°C for a period of 24h respectively. After incubation the curd was mixed properly to break large curd particles and poured in to 50ml autoclaved plastic tubes and centrifuged at 10,000 rpm/min at 4^0C using Kubota High speed centrifuge (Japan). The supernatant obtained after filtration with 0.45µm filter was lyophilized and used with minimum amount of distilled water when required.

Gel filtration

Readymade Gel filtration column 5ml was purchased from Banglore Geni. The sample 2-5% of bed volume was loaded and eluted with double glass-distilled water. Double the bed volume number of fractions 1ml each was collected (discarded the first 5ml). The presence of peptide determined by taking the absorbance at 340nm Fig 1.1.The peaks obtained in G-25 chromatogram were again lyophilized and dissolved in minimal amount of double glass distilled water. Final concentration of peptide in test sample was measured using formula given below.

Sample concentration =_OD of Unknown × Concentration of known Standard/ OD of Sample

HPLC conditions

The reverse phase HPLC (Waters, USA) with Spherisorb C-18 column (4.6 X 250mm) with 20µl loop was used for the separation of the peptides. Gradient solvent delivery was achieved using two Water's pumps at the flow rate of 0.75 ml/min. Solvent A was 0.1% Trifluoroacetic acid (TFA) in HPLC (Milli-Q) grade water. Solvent B was 0.09% TFA, 90% acetonitrile. Both the solvents were filtered using 0.45µm membrane filters and degassed before use. The C-18 column was thoroughly washed with solvent until the base line was obtained. Twenty µl of the sample was injected. Detection was monitored with Water's dual detector at 220 nm and 280 nm for all the fractions. The fractions of the respective peaks was pooled and lyophilized.

Preparation of peptide dilution range

Sterile test tubes were used to prepare dilutions to conduct the test. Tubes were closed with plastic or metal closure caps. Final dilution of peptides was made volumetrically in the broth. A volume of 100 µL, 50 µL, 30 µL, 20 µL, 10 µL, 5 µL of each dilution was used for the test.

Preparation of Inoculums of test culture

Inoculums of test culture was prepared by making a direct broth suspension of isolated colonies of *E. coli* and *Staphylococcus aureus* selected from an 18- to 24-hour agar plate. Test cultures were taken from reference culture of our lab. Suspension was made to achieve a turbidity having 1 x 10^6 microorganisms after 18 to 20 hour period of incubation at 37^0C.

Results and Discussion

The aim of broth and well diffusion methods is to determine the lowest concentration of the assayed antimicrobial agent i.e. peptide, that under defined test conditions, inhibits the visible growth of the bacterium being investigated. Broth dilution and agar diffusion method was used to find out the effectiveness of isolated peptide.

In Broth dilution method

Fresh culture of *E. coli* and *Staphylococcus aureus* was used in the test. These were inoculated into nutrient agar broth medium in the presence of different concentrations of peptide separately. Two ml of nutrient broth was taken in each tube and these were inoculated with 100µL, 50µL, 30µL, 20µL, 10µL, 5µl of peptides. Control tube was containing only broth, after mixing tubes were incubated at 37ºC for 24 hours in triplicates. Growth was measured after incubation (Fig 3.1 and Fig 3.2).

Optical density (OD) of control and other sample were taken after 24hr and 48 hr respectively to determine MIC value of peptide. The broth culture was plated on agar surface to count the number of colonies in terms of cfu/ml. Table 3.1 shows that when *E. coli* was added in nutrient broth medium and incubated for 24 hours, OD was taken as 1.52 and 100µl peptide was added and incubated for another 24 hours, OD was 1.00 and 6.2 $\times 10^6$ cfu per ml was count, second tube was added with 50µl peptide was inoculated and OD was 1.48 and colony count was 2 $\times 10^4$ cfu/ml followed by 30µl peptide inoculation with 1.63 as OD and 9.6 $\times 10^5$ cfu/ml was colony count. In 20µl peptide inoculation, OD was 1.70 and 2 $\times 10^5$ cfu/ml was colony count followed by 10µl peptide inoculation with OD was taken as 1.77 and 5.4 $\times 10^6$ cfu/ml as colony count and 5µl peptide inoculation shows 1.78 as OD and 6 $\times 10^6$ cfu/ml colony count. Hence, Peptide is effective in showing antimicrobial property up to 20µl peptide inoculation.

When *Staphylococcus aureus* was added in nutrient broth medium and incubated for 24 hours with OD was taken as 1.45 and 100µl peptide was inoculated and incubated for another 24 hours, OD was 1.62 and count 9.2 $\times 10^4$ cfu/ml was calculated. In second sample, 50µl peptide was inoculated and OD was 1.5 and colony count was 4.2 $\times 10^5$ cfu/ml followed by 30µl peptide inoculation with 1.54 as OD and 5.1 $\times 10^5$ cfu/ml colony count. In 20µl peptide inoculation, OD was 1.60 and 6 $\times 10^6$ cfu/ml was colony count followed by 10µl peptide inoculation with OD was taken as 1.63 and 4 $\times 10^6$ cfu/ml colony count (table 3.2). For both *E. coli* and *Staphylococcus aureus* OD was decreased as amount of peptide was increased. The inhibition was seen up to addition of 20µl peptide and after that there was no inhibition of growth as evidenced by cell count cfu/ml after plating the suspension on an agar plate as seen in Fig 3.3.

Agar well diffusion method

In Well diffusion method nutrient agar was prepared and plated under aseptic condition.

Table.1.1 Showing concentration of Peptides in unknown sample.

S.No.	Volume of Peptides	OD at 340 in triplicate			Mean values of OD	Concentration of peptide (mg/ml)
1.	100 µl	0.19	0.19	0.20	0.19	1.67
2.	50 µl	0.08	0.09	0.09	0.08	0.68
3.	30 µl	0.07	0.06	0.06	0.06	0.51
4.	20 µl	0.04	0.04	0.04	0.04	0.34
5.	10 µl	0.03	0.02	0.02	0.02	0.17
6.	5 µl	0.01	0.01	0.02	0.01	0.08

Table.3.1 Showing colony count of E. coli incubated with different amount of peptide

Sample	OD after 24 hours	Addition of Peptide	OD after 48hours	Colony Count (per ml)
Control	1.52	-----	1.78	6.2×10^6
1	1.52	100ul	1.00	3.0×10^3
2	1.54	50ul	1.48	2.0×10^4
3	1.51	30ul	1.63	9.6×10^5
4	1.52	20ul	1.70	2.0×10^5
5	1.52	10ul	1.77	6.0×10^6
6	1.51	5ul	1.78	6.0×10^6

Table.3.2 Showing colony count of *Staphylococcus aureus* incubated with different amount of peptide

Sample	OD after 24 hours	Addition of Peptide	OD after 48hours	Colony Count (per ml)
Control	1.46	-----	1.62	2.2×10^6
1	1.45	100 ul	1.33	9.2×10^4
2	1.43	50ul	1.5	4.2×10^5
3	1.46	30ul	1.54	5.1×10^5
4	1.46	20ul	1.60	5.6×10^5
5	1.46	10ul	1.63	4×10^6
6	1.45	5ul	1.63	4×10^6

Table.3.3 Inhibition of *Staphlococcus aureus* and *E.coli* by inoculation with different peptide concentration generated during fermentation with *L.helveticus*.

S.No.	Concentration of Peptides	Zone of Inhibition Diameter (mm)					
		Staphyloccus aureus			***E.coli***		
1.	100 µl	14	16	16	15	17	17
2.	50 µl	11	12	11	14	12	12
3.	30 µl	9	9	9	11	11	10
4.	**20 µl**	**8**	**8**	**10**	**9**	**9**	**9**
5.	10 µl	-	-	-	-	-	-
6.	5 µl	-	-	-	-	-	-

Fig.3.1 Showing inhibition of E. coli after addition of peptide in broth culture.

Fig.3.2 Showing inhibition of *Staphylococcus aureus* after addition of peptide in broth culture.

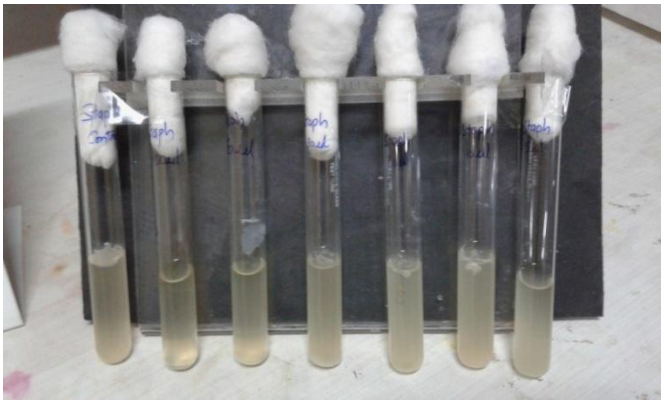
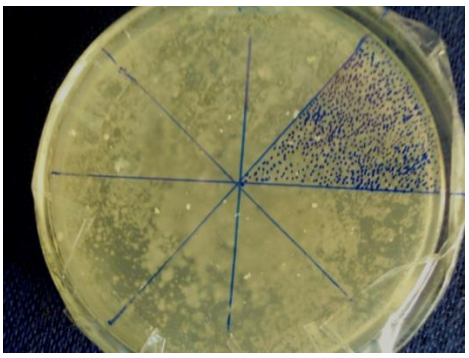
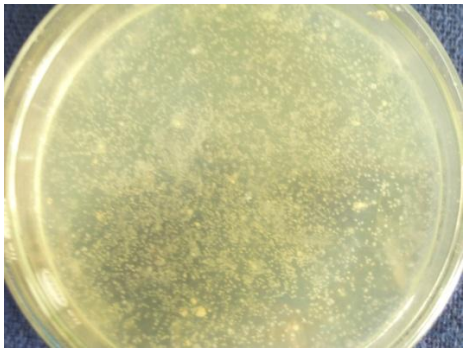


Fig.3.3 Plate showing colony count for Staphyloccocus with and without peptide inoculation

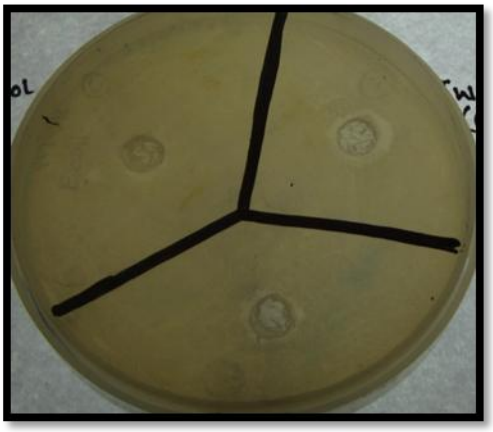


Control

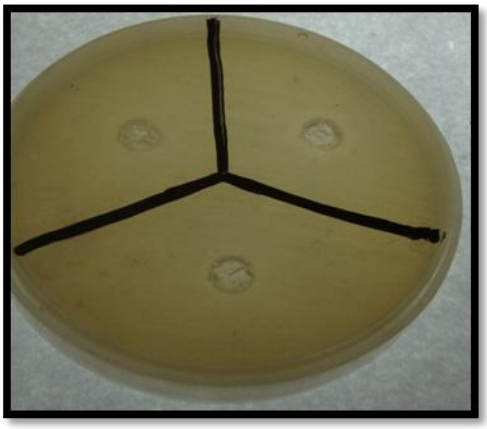


with 20ul peptide inoculation

Fig.3.4 Showing inhibition of E. coli and Staphylococcus by 20µl peptide inoculation.



20µl peptide on *E. coli* lawn



20µl peptide on Staphylococcus lawn

Using 7mm diameter agar medium well cutter wells were made at equal distance. A drop of the soft agar was dropped into the well to the seal the bottom. The test organism *E.coli*, *Staphylococcus aureus* swabbed on the respective plates. After allowing for 10 min setting, 100 µl of peptide was added into one of the well and sterile distilled water to another well which is used as a control and 50µl, 30µl, 20µl 10µl and 5µl of peptide was added to respective wells in plates. These plates were incubated without inverting at 37ºC for 24 hours in triplicate. Peptide diffusion from these sources into the agar medium leads to the inhibition of bacterial growth in the vicinity of the source and to the formation of clear zones without bacterial growth.

Table 3.3 and Fig-3.4 shows 16mm zone of inhibition against *Staphylococcus aureus* when 100µl peptide was added followed by 17mm against *E. coli*. About 12mm zone of inhibition found when 50 µl of peptide concentration was used against *S. aureus* and 12 mm zone against *E. coli.* Plates containing 30µl of peptide concentration showed 9mm Zone of inhibition against *S. aureus* and 11 mm against *E. coli*. There was no inhibition seen when 10µl and 5µl of

peptide was added to the wells. Hence it is clear that 20µl of suspension containing amount of peptide was effective minimal concentration to for test organisms as observed in tube dilution and agar well diffusion method.

In conclusion, for a test to be considered valid, acceptable growth ≥ 2 mm button or definite turbidity must occur in the growth-control well. The lowest concentration at which the isolate is completely inhibited as evidenced by the absence of visible bacterial growth was recorded as the minimal inhibitory concentration or MIC. By Comparing the amount of growth in the wells or tubes containing the peptide with the amount of growth in the growth-control wells or tubes used in each set of test., hence the study demonstrated that bioactive peptides generated during fermentation with *L. helveticus* showed a minimum of 8mm zone of inhibition against gram positive *S. aureus* and 9mm against gram negative *E. coli* on plates containing 20µl peptide concentration as compared to respective plates or broth tubes which determines the lowest concentration of the assayed antimicrobial peptide will be minimal inhibitory concentration, MIC.

This study shows that higher the amount of peptide higher the inhibition. As evidenced by plates containing 20µl peptide concentration as compared to respective plates and broth tubes determine the lowest concentration of the assayed antimicrobial peptide i.e. minimal inhibitory concentration (MIC) because after 20µl i.e. 10µl and 5µl amount of peptide was found to have insufficient concentration of peptides to inhibit the growth of pathogens. Thus the amount of peptide concentration in 20µl of peptide suspension will be considered as effective minimal inhibitory concentration. The amount of peptide as calculated for 20µl peptide suspension was 0.34 mg/ml (Table-1.1). Therefore we can conclude that 0.34 mg/ml of may be considered as minimal inhibitory concentration for the inhibition of growth of *S. aureus* and *E. coli.* Further study and animal trials must be carried out to decide final dose of such peptide to be used for therapeutic agent or as in food preservation.

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