



## ANTIBACTERIAL ACTIVITY OF PAPAIN HYDROLYSATES OF BUFFALO MILK WHEY PROTEIN AGAINST MASTITIS PATHOGENS

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### ABSTRACT

Mastitis, a severe infectious disease among dairy cattle, is still a challenge for livestock treatment due to the increased infections by antibiotic resistant strains. Antimicrobial peptides could be the alternate for treating such drug resistant strains. In the present study, the whey proteins extracted from buffalo milk was subjected to enzymatic hydrolysis using trypsin, chymotrypsin, pepsin, papain and Proteinase K. Antibacterial assay carried out with these hydrolysates against *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* isolated from mastitis milk showed that these hydrolysates have an inhibitory effect on their growth. Papain hydrolysates showed highest antibacterial activity against *E.coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*. The fraction 4 (12 min fraction) of RP-HPLC purified papain hydrolysate showed highest antibacterial activity and that fraction has got Minimum inhibitory concentration of 100 µg/ml against *E.coli* and 150 µg/ml against *Staphylococcus aureus*, 100 µg/ml against *Streptococcus agalactiae* and 100 µg/ml against *Streptococcus dysgalactiae*.

**KEY WORDS:** Whey protein, Buffalo milk, antibacterial activity, Papain hydrolysate



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## INTRODUCTION

Mastitis is a microbial infection of udder of the livestock which results in a decline in health of animal as well as high load of infectious organisms in milk which affects the quality. Treatment of mastitis remains as a challenge till today because of antibiotic resistant strains. The long term use of antibiotics has resulted in multi-drug resistant strains of bacteria and hospitals have become breeding grounds for human-associated microorganisms<sup>1</sup>. There is an urgent need to find alternatives to synthetic antibiotics. The two classes of antimicrobial peptides, non-ribosomally and ribosomally-synthesized peptides provided a new therapeutic strategy to fight microorganisms<sup>2-4</sup>. The discovery of antimicrobial peptides makes natural antibiotics the basic element of a novel generation of drugs for the treatment of bacterial and fungal infections<sup>5, 6</sup>. The broad spectrum of antimicrobial activities reported for these molecules suggests the potential benefit in the treatment of cancer<sup>7</sup>, viral<sup>8</sup> and parasitic infections<sup>9</sup>. Antimicrobial peptides were reported to have potent activity against fungi like *Candida*<sup>10</sup>. Antibacterial peptides are encrypted within the sequence of milk proteins. These encrypted antibacterial peptides can be released by hydrolysis of the milk protein. Antibacterial peptides have been derived from the bovine milk proteins, lactoferrin,  $\alpha_{S1}$ -casein<sup>11</sup>,  $\alpha_{S2}$ -casein<sup>12</sup>,  $\alpha$ -lactalbumin,  $\beta$ -lacto globulin and k-casein<sup>13</sup>. In India much work has not been done on the production of antibacterial peptides from buffalo milk proteins. No work has been reported for antibacterial peptides from buffalo milk proteins. The present study was undertaken to hydrolyze the buffalo milk whey protein by using trypsin, pepsin, Chymotrypsin, proteinase K and papain and to study the antibacterial activity of the hydrolysates.

## MATERIALS AND METHODS

### (i) Sample collection

Milk samples from Murrah buffaloes (*Bubalus bubalis* L.) were collected from Livestock

Research Station, Kattupakkam for whey protein extraction. The bacteria were isolated from milk sample collected from mastitis infected cows from Madras Veterinary College, Chennai.

### (ii) Whey Protein Extraction

The fat content of the milk was removed by centrifuging the milk samples at 5000 rpm for 15 minutes. To the skimmed milk, 1N Hydrochloric acid was added in drops to precipitate casein<sup>14</sup>. The separation took place at a pH of about 4.6. The precipitated casein was separated out from whey by filtering using Whatmann No.1 filter paper. The filtered whey was again centrifuged at 5000 rpm for 15 minutes to remove any traces of casein, if present. The whey protein thus obtained was stored at -20°C for future use.

### (iii) Protein Estimation

The protein content in the sample was estimated using Biuret's method. Bovine Serum Albumin (BSA) was used to prepare the standard curve.

### (iv) Enzymatic Hydrolysis of Whey Protein

Three digestive enzymes, trypsin, chymotrypsin and pepsin, one plant-derived enzyme, papain and proteinase K were used to hydrolyze the whey protein.

### (v) Trypsin, Chymotrypsin and Papain Digestion

Trypsin, chymotrypsin and Papain hydrolysis were carried out after adjusting the pH of the sample to 8.0 and at a temperature of 37°C in dry bath. The optimized enzyme-substrate ratios employed for digestion was 1:50. The hydrolysis was carried out for a period of 4 hours.

### (vi) Pepsin Digestion

Pepsin hydrolysis was carried out 37°C and pH was adjusted to 2.0 using 1N HCl. The enzyme-substrate ratios used was 1:50 and hydrolysis was carried out for 4 hours.

**(vii) Proteinase K Digestion**

Proteinase K hydrolysis was carried out 37°C and pH was adjusted to 7.0. The enzyme-substrate ratio used was 1:50 and hydrolysis was carried out for 4 hours.

**(vii) Lyophilization**

The digested protein sample was then lyophilized using a freeze dryer for 8 hours. The lyophilized sample was then stored at -20°C for future use.

**(viii) Antibacterial assay**

Bacteria were isolated from milk of mastitis infected cow and four different isolates were examined and identified as *E.coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*<sup>15</sup>. The antibacterial activity of the hydrolysates was tested against bacterial isolates such as *E.coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* using well-diffusion technique<sup>16</sup> with slight modifications. 100 µl (2mg/ml) of hydrolysates of whey protein by pepsin, trypsin, chymotrypsin, papain and proteinase K were used for testing the antimicrobial activity. The plates were then incubated overnight at 37°C and the plates were examined for the presence of zone of inhibition. Papain hydrolysates showed the highest zone of inhibition. So papain hydrolysates were taken for further analysis.

**(ix) Purification of antibacterial peptides by RP-HPLC**

Initial fraction of the Papain hydrolysates of whey protein was achieved using preparative reverse phase chromatography (RP-HPLC). A preparative C-18 column was used with a Hewlett-Packard 1050 series HPLC equipped with an HP automated chemstation and UV array detector, all eluates were monitored at 254 nm. The solvents consisted of: (A) 0.1% (v/v) trifluoroacetic acid (TFA) in deionised water and (B) 0.1% (v/v) TFA in acetonitrile. After injection of the samples, the HPLC system (flow rate of 2 mL min<sup>-1</sup>) was equilibrated with 95% solvent A for 5 min, followed by a linear

gradient of 5-45% (v/v) solvent B over 80 min to elute peptides, and a 5 min re-equilibration with 95% (v/v) solvent A. Further fraction of the Papain hydrolysates of whey protein and re-chromatography of purified peptides was performed using an analytical C-18 column compatible with MS detection and connected to the HPLC. The column temperature was maintained at 45°C using a HPLC column heater. The solvent consists of: (A) 0.02% (v/v) TFA in deionised water and (B) 0.02% (v/v) TFA in acetonitrile. After injection of samples onto the column, the HPLC system was operated at a flow rate of 1 mL min<sup>-1</sup> under the linear gradient and equilibration conditions described above. The fractions from RP-HPLC were collected at programmed time intervals using a fraction collector<sup>17</sup>.

**(x) Antibacterial Assay of HPLC purified samples using well-diffusion method**

**Antimicrobial activity analysis**

100 µl of HPLC purified lyophilized fraction (0.5mg/ml) were loaded onto the wells. The plates were then incubated overnight at 37°C and the plates were examined for the presence of zone of inhibition. All the fractions collected from HPLC were tested for antibacterial activity and fraction 4 (12minutes) of papain hydrolysates showed highest antibacterial activity and this fraction has been taken for Minimum inhibitory concentration determination. Minimum inhibitory concentration determination of

**HPLC purified samples using 96 well micro titre plates**

The HPLC purified samples were Lyophilized and dissolved in deionized water (500µg/ml). It was used to screen the antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* using a modification of the critical dilution method<sup>17</sup>. The antibacterial assays were performed in sterile 96-Well microtitre plates by the method<sup>18</sup>. PEP broth was used for growth of *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* and the test sample was prepared

with growth medium (50µl), HPLC purified fraction (10µl) in different concentration (25µg/ml, 50µg/ml, 75 µg/ml, 100 µg/ml, 125 µg/ml, 150 µg/ml, 175 µg/ml and 200 µg/ml) and bacterial inoculum (5 µl of overnight broth culture diluted to approximately  $5 \times 10^6$  CFU mL<sup>-1</sup> in sterile 10% (w/v) peptone for *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*). Controls made with sterile water instead of the peptide solution and the standard containing amikacin instead of peptide solution. Three replicates were maintained for each sample. The initial O.D was taken at 0 hour and intermittently for every two hours at 600 nm for 24 hours. The mixture was maintained at 37°C.

## RESULTS

### 1. Protein Estimation

The whey protein concentration was estimated by Biuret's method. From the standard curve plotted, the protein content was estimated to be 4.5 mg/ml.

### 2. Anti-microbial Assay

The hydrolyzed samples were analyzed for their anti-bacterial activity against isolates such as *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* and the zone of inhibition measured in mm is given in Table 1.

**Table 1**

**Zone of inhibition in mm produced by enzyme hydrolysates of whey protein against *E.coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*.**

Bacterial isolates	Zone of inhibition (in mm) produced by hydrolysates (2mg/ml) of whey protein						K
	Control Amikacin 30µg	Trypsin hydrolysates	Chymotrypsin hydrolysates	Pepsin hydrolysates	Papain hydrolysates	Proteinase hydrolysates	
<i>Escherichia coli</i>	17.5±0.30	9.4±0.15	7.6±0.15	7.3±0.30	14.5±0.30	2.5±0.12	
<i>Staphylococcus aureus</i>	14.5±0.20	10.3±0.25	5.4±0.46	10.1±0.42	15.4±0.32	5.2±0.15	
<i>Streptococcus agalactiae</i>	18.3±0.30	8.4±0.20	7.5±0.10	8.5±0.15	14.4±0.20	4.5±0.21	
<i>Streptococcus dysgalactiae</i>	15.6±0.28	11.1±0.28	8.5±0.50	8.6±0.57	15.1±0.76	6.3±0.57	

Values are mean of triplicates ±SD

It was observed that the papain hydrolysates showed highest inhibitory activity, 14.5±0.30mm against *E. coli*, 14.4±0.20mm against *Streptococcus agalactiae*, 15.4±0.32mm against *Staphylococcus aureus* and 15.1±0.76mm against *Streptococcus dysgalactiae*. Pepsin and Chymotrypsin showed least inhibition against *E. coli* and chymotrypsin showed least inhibition against *Staphylococcus aureus*. Trypsin had shown 9.4±0.15mm, 10.3±0.25mm, 8.4±0.20mm and 11.1±0.28mm against *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* respectively.

### 3. Antibacterial activity of RP- HPLC purified Lyophilized samples using Well Diffusion Method

The RP-HPLC purified lyophilized fractions were tested against *E. coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* by agar well diffusion method. Of all the fractions, Fraction 4 collected at 12 minutes have shown the maximum activity of 13.6±0.15mm, 14.5±0.30mm and 13.3±0.30mm against these bacteria. The other fractions have not produced significant antibacterial activity.

#### **4. Minimum inhibitory concentration determination of papain hydrolysates using 96 well titre plate method**

The minimum inhibitory concentration was found to be 100µg/ml against *E. coli*, 150 µg/ml against *Staphylococcus aureus*, 100µg/ml against *Streptococcus agalactiae* and 100µg/ml against *Streptococcus dysgalactiae*.

## **DISCUSSION**

The purified casocidin obtained by chymosin digestion of casein at neutral pH have been reported to have activity against *Staphylococcus*, *Sarcina*, *Bacillus subtilis*, *Diplococcus* and *Staphylococcus pyogenes*<sup>11</sup>. Antibacterial activity of bovine lactoferrin derived peptide has been well emphasized<sup>18</sup>. Antihypertensive activity of a tripeptide derived from β-lactoglobulin has been reported<sup>19</sup>. The hydrolysed bovine casein with range of proteolytic enzymes pepsin, trypsin, alpha chymotrypsin, beta chymotrypsin and reported antibacterial activity of peptide cp1 and cp2 corresponding to residues of bovine αS1 casein (99-109 residues) and αS2 casein(residues 183-207)<sup>20</sup>. β-lactoglobulin from buffalo (*Bubalus bubalis*) milk whey was characterized and its interaction with erythrocyte lipocalin

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interacting membrane receptor has been previously studied<sup>21</sup>. There are no reports for peptides from buffalo milk whey protein. In the present study the Papain hydrolysates of buffalo milk whey protein was found to be having antibacterial activity against *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*.

## **CONCLUSION**

Papain hydrolysates of whey protein from buffalo milk sample have potent antibacterial activity against mastitis pathogens such as *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae*. The fraction 4(12 min fraction) of RP-HPLC purified papain hydrolysate showed highest antibacterial activity and that fraction has got Minimum inhibitory concentration of 100 µg/ml against *E. coli* and 150 µg/ml against *Staphylococcus aureus*, 100 µg/ml against *Streptococcus agalactiae* and 100 µg/ml *Streptococcus dysgalactiae*. The study revealed that whey protein from buffalo milk can be used for production of antibacterial peptides and can be used for treating bacterial infections after evaluating their antibacterial activity under *in vivo* condition.

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