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Isolation and detection of low molecular weight cationic peptide in milk of mastitic buffaloes

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ABSTRACT

Milk samples from mastitic and healthy buffaloes were subjected to centrifugation for preparation of acellular milk. It was subjected to ion exchange chromatography using carboxy methyl resin for purification. The purified protein and peptides obtained by the ion exchange chromatography were subjected to acid urea-polyacrylamide gel electrophoresis using lysozyme as a marker. The milk samples from mastitic buffaloes contained many cationic proteins and peptides. Some of these peptides exhibited migration similar to lysozyme. The SDS-PAGE revealed the presence of low molecular weight peptides in range of ~3–5KDa in mastitic milk, whereas these low molecular weight peptides were absent in milk from healthy animals. Tricine SDS PAGE was used for the resolution of peptides using high concentration of Acryl amide (49.5%) for resolving proteins less than 5KDa. It also revealed the presence of low molecular weight peptide of ~3–4.5KDa in mastitic milk and absence of such peptides from normal healthy buffalo's milk. The present work has detected the presence of low molecular weight cationic peptides in milk of mastitic Buffaloes.

Key words: AU-PAGE, Cationic peptides, Ion exchange chromatography, Mastitis, SDS-PAGE, Tricine SDS-PAGE

The peptide based antimicrobial defense is an evolutionarily ancient mechanism of host defense, found in both the plant and animal kingdoms (Boman *et al.* 1991). Several types of antimicrobial peptides (AMP's) especially α-defensins have been found in epithelial layers, phagocytes and body fluids of eukaryotes indicating their importance in the innate immune system (Hancock and Diamond 2000). Present knowledge on the innate immune defense of buffalo is less comprehensive as compared to that of mice, cattle and human beings. Hence, the aim of present study was to isolate and detect the presence of low molecular weight cationic peptide in the buffalo milk so as to determine, if these peptides play any role in the innate immune defense during mammary gland infection.

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MATERIAL AND METHODS

Buffaloes (20) suffering from mastitis (as indicated by clinical symptoms and California Mastitis test) were selected from Dairy Complex Haibowal and from cases presented at clinical services complex, of the university. The samples were subjected to microbiological culture examination and only the samples positive for bacterial infection were used in the present study. Control group constituted 10 numbers of apparently healthy buffaloes which were selected from Dairy farm of GADVASU, Ludhiana.

Sample collection

An approximate quantity of 30 ml of each mastitic milk sample was collected from all the affected animals and brought to laboratory under ice cold conditions, for further processing. A similar quantity of normal buffalo milk samples were also collected in sterilized glass vial, and stored at –20°C till further processing.

Preparation of acellular milk

The mastitic milk was subjected to centrifugation at 200 g at 4°C for 20 min. The upper milk fat layer was separated out and further centrifuged at 27000 g at 4°C for 30 min. A quantity of 20 ml of acellular milk so obtained was transferred to a clean

sterilized glass beaker and then mixed with 4–5 ml of CM (carboxy methyl) weak cation exchanger slurry. The equilibration was carried out with 0.5% (v/v) acetic acid pH 7.5, with volume 4–5 times slurry used and mixed intermittently with the help of magnetic stirrer for about 45–60 min. The slurry was kept at room temperature for another 60 min and then transferred to refrigerator for overnight incubation.

Purification of peptides by ion exchange chromatography

The method described by Valore *et al.* (1998) was followed with slight modification. The settled beads were poured into the column and washed with 25 mM ammonium acetate (pH 7.5), 4–5 times the volume of the slurry used, until stable absorbance at 280 nm was achieved. The washing flow-through was collected at 30 sec interval for 2 min and monitored by taking the absorbance reading at 280 nm. Then the absorbed protein and peptides were eluted with 10 ml of 5% glacial acetic acid. The column effluent was monitored at 280 nm and 4 ml fractions were collected and checked for absorbance each time in UV spectrophotometer. The peak protein fractions so obtained were pooled and stored at –20°C. The eluted proteins were subjected to Expander concentrator 5301 (England), for concentration and also they were concentrated by using technique of Dialysis.

Detection of cationic peptides by Acid Urea PAGE

The presence of cationic peptides was detected by carrying out Acid Urea Polyacrylamide gel electrophoresis (AU-PAGE) as described by Selsted *et al.* (1992) with slight modification. For separating small cationic peptides, 15% gel was preferred. AU-PAGE is specifically used for separation of cationic proteins and peptides.

After the preparation of the gel, it was subjected to pre-electrophoresis for about 2 hr at 4°C at constant 150 V. The gel was run in reverse polarity (red to black). Electrophoresis was carried out at constant current mode of 5 mA/slot so that cationic peptides moved downwards towards the cathode. The gel was stained with CBB dye and destained with destaining solution containing 50% of Propanol and 10% of glacial acetic acid.

Determination of molecular weight by SDS PAGE

The molecular weight of the cationic peptide was determined by using SDS PAGE method as per Sambrook *et al.* (1989). The electrophoresis was carried out at room temperature at 60 V initially till the stacking portion, afterwards changed to constant voltage of 100V for resolving. The gel was run until the tracking dye front reach the end of the glass plates, 1 cm above the end margin. The gel was visualized by with Coomassie Brilliant blue R–250 and Silver staining as per Bassam *et al.* (1991). Molecular weight was compared using standard ultra colour low molecular weight marker using gel documentation.

Tricine SDS PAGE for determination of small molecular weight peptides

Tricine SDS PAGE was used as per Schagger and Von Jagow (1987) for determination of small molecular weight peptides. This form of electrophoresis is preferred for separation of proteins of lower molecular weight. A gel gradient of 16% gel or 16% urea was overlaid directly with 10% gel about 1 cm above it, and kept for polymerization. It was directly overlaid with 4% stacking gel. Electrophoresis was carried out at constant current mode of 5 mAm/slot. The gel was visualized by with Coomassie Brilliant blue R-250 and Silver staining as per Bassam *et al.* (1991). Molecular weight was compared using standard ultra colour low molecular weight marker using gel documentation.

RESULTS AND DISCUSSION

Peptides purification by ion exchange chromatography

The milk samples after subjecting to ion exchange chromatography, it was observed that there was increase in absorbance at 280 nm just after treating with the elution buffer solution, which indicated the presence of cationic proteins and peptides in milk samples. The peak obtained in case of milk from healthy animals was comparatively smaller. There was significant difference in the level of protein eluted from mastitic milk samples as compared to milk from normal healthy animal (Table 1).

Table 1. Protein eluted from mastitic milk

Test (n=15)		Control (n=10)	
Mean OD at 280nm (last washing)	Mean OD at 280nm (1st peak eluate)	Mean OD at 280nm (last washing)	Mean OD at 280nm (1st peak eluate)
1.423±0.2798[a]	2.512±0.3600[b]	2.959±0.4265[A]	2.580±0.4599[A]

There is significance difference in mean O.D. value of within test group as compare to control group as shown by applying t-test.

The methodology adopted in present study was found to yield the desired peak fraction containing cationic peptide. Valore *et al.* (1998) have also used ion exchange chromatography technique using the CM Macro-prep beads (cation exchanger) to isolate and purify cationic peptide from urine, blood, tissues and vaginal lavage and the result are consistent with the finding of present study. Similarly, Yarus *et al.* (1996) have also used Ion exchange chromatography for purification of bTAP (bovine tracheal antimicrobial peptide). Similarly, Das *et al.* (2008) have reported an increase in the levels of cationic antimicrobial peptides which was apparent in milk obtained from natural cases of mastitis, as compared to the levels in normal milk. The present study detects the presence of cationic proteins and peptides in the mastitic milk, which are in higher concentration than milk from healthy animals. The success of CM Macroprep in

separation of cationic proteins and peptides from the milk was also confirmed.

Acid urea polyacrylamide gel electrophoresis

The various mastitic milk samples were resolved into 6–16 bands (Figs 1, 2). The electrophoregram showed the presences of cationic proteins/peptides are having mobility similar to lysozyme, which has been used as standard. Lysozyme is highly cationic antimicrobial peptide present in saliva and other secretion of the body. The level of cationicity of a protein/peptides is related to the ability to bind to microbes which have negatively charged membranes. The present study confirmed the presence of cationic proteins and peptides in the buffalo acellular milk.

The literature is inconsistent regarding the number of cationic proteins and peptides in domestic animals based on AU-PAGE. Various workers have reported varied number of protein bands in different species. The number of bands reported in different species ranged from 10 to 15 (Gennaro *et al*. 1984, Selsted *et al*. 1993; and Sarmah and More 1993). The present study showed concordance with observations of previous workers. The further studies need to be undertaken to characterize each peptides individually.

SDS PAGE analysis of cationic peptides Low molecular weight peptides (3–4 KDa) in mastitic milk sample from buffaloes were observed (Fig3), whereas no such peptides were observed in the milk from healthy animals (Fig 4). Gennaro *et al.* (1984) reported about 10 bands in 10–20% gel gradient in SDS PAGE of molecular weight of 13 KDa to 90 KDa. Marzari *et al*. (1998) reported several small molecular weight proteins from 1.6 to 25 KDa which originated from a single larger precursor protein by post translational processing. Sarmah (1989) and Singh (1993) reported that the molecular weights of buffalo PMN's granular cationic proteins and peptides ranged from 3 KDa to 80 KDa. Selsted *et al.* (1992) also reported the presence of antimicrobial peptides from the mouse intestines which are in molecular range of ~ 4 KDa.

The defensins have been found to be consisting of 38–42 amino acids and having the molecular weight in the range of less than 5 KDa. The presence of low molecular weight peptides in the similar range indicates the possibility of expression of defensins in the inflamed tissues.

Tricine SDS PAGE analysis of cationic peptides

Low molecular weight peptides in mastitic milk sample of buffaloes could be observed (Fig. 6), which were absent in milk from healthy animals (Fig. 5 a,b). These peptides corresponded to molecular weight of ~3KDa. In the present studies high concentration of Acrylamide (49.5%) along with Tris- Tricine buffer was used to resolve proteins <5KDa. With this protocol 10–16 protein bands were visible. Peptides of low molecular weight of ~3–4.5 KDa were obtained from the milk of mastitic buffaloes whereas the normal healthy milk didn't express any such peptides. This indicates that these peptides are inducible in nature upon challenge with microbes and inflammatory cytokines. These peptides may be found not only in the gland but also in milk (Zhang *et al.* 2000). Thus mammary antimicrobial peptides might protect the surface of the secretary tissues of the mammary gland against infection and through milk to be transferred to the offspring, thus increasing its resistance.

The present studies revealed the presence of peptides which are cationic in nature having the same mobility as that of lysozyme and having molecular weight in range ~3–4KDa. Further work is warranted on sequencing of these

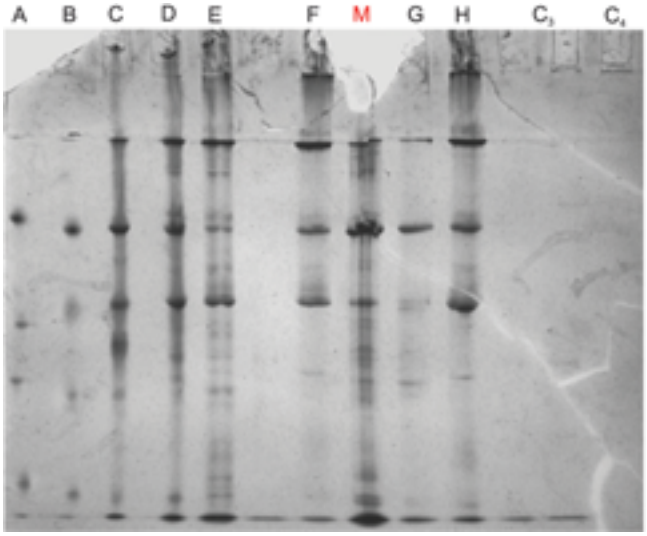


Fig. 1. ACID-UREA PAGE-based electrophoregram of milk samples subjected to ion exchange chromatography.A, B, C, D, E, F, G, H, I- samples from milk of mastitic buffalo, C_1 and C_2 samples from control healthy milk of Buffalo,M- Lysozyme standard

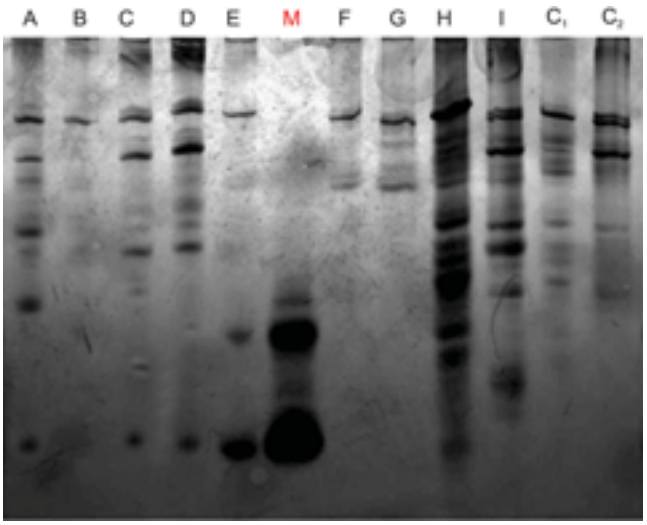


Fig. 2. ACID-UREA PAGE-based electrophoregram of milk samples subjected to ion exchange chromatography.A, B, C, D, E, F, G, H, I samples from milk of mastitic buffalo, C_3 C_4, samples from control healthy milk of buffalo, M- Lysozyme standard

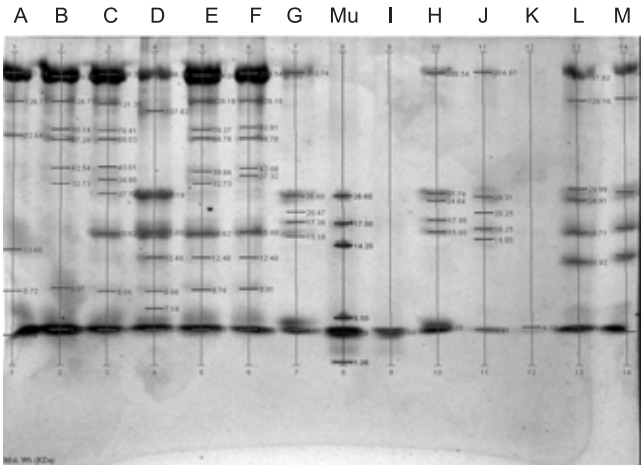


Fig. 3. SDS-PAGE-based electrophoregram of milk samples subjected to ion exchange chromatography.A, B, C, D, E, F, G, I, H, J, K, L, M samples from milk mastitic of buffalo. Mu: ultra colour marker for low molecular range (M.W.1060-26600KDa)

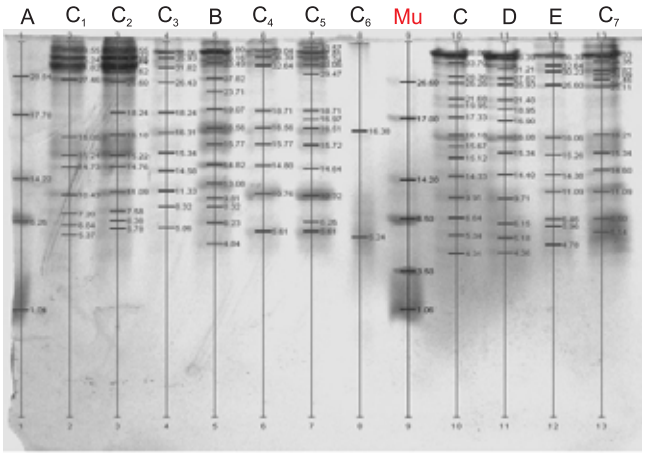
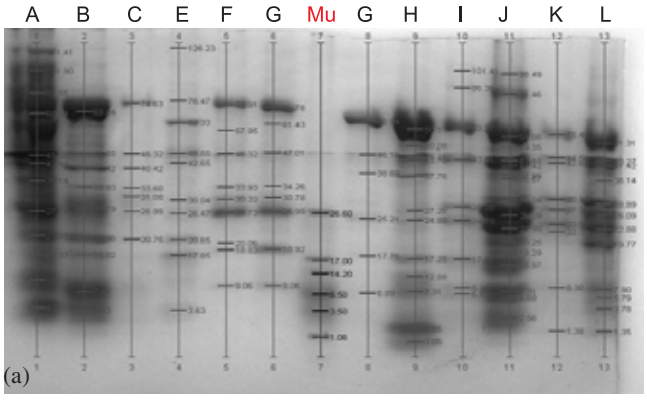


Fig. 4. SDS-PAGE based electrophoregram of milk samples subjected to ion exchange chromatography.C_1, C_2, C_3, C_4, C_5, C_6, C_7, samples from control healthy milk of buffalo, B, C, D, E samples from milk of mastitic buffalo. Mu: ultra colour marker for low molecular range (M.W.1060-26600 KDa)



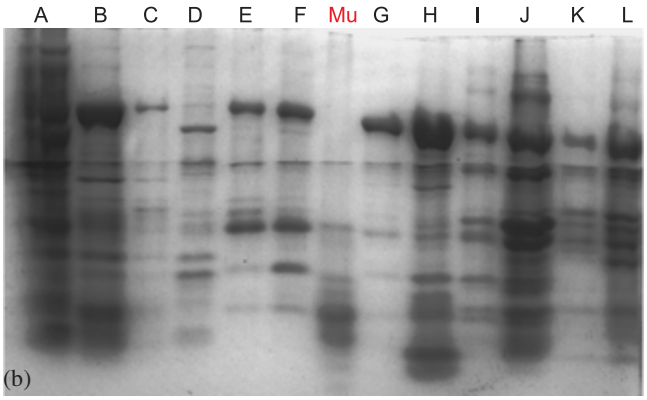


Fig. 5(a): Tricine SDS PAGE-based electrophoregram of milk samples subjected to ion exchange chromatography.A, B, C, D, E, F, G, H, I, J, K, L, M samples from milk of mastitic buffalo. Mu: ultra colour marker for low molecular range (M.W.1060-26600KDa) (b): Tricine SDS PAGE-based electrophoregram (unmarked) of milk samples subjected to ion exchange chromatography (silver staining).A, B, C, D, E, F, G, H, I, J, K, L, M samples from milk of mastitic buffalo. Mu: ultra colour marker for low molecular range (M.W.1060-26600KDa)

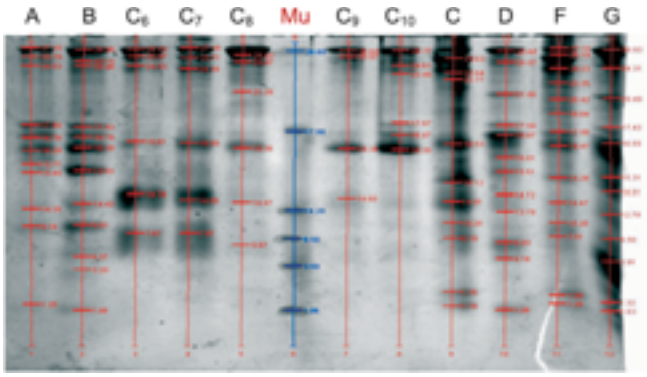


Fig. 6. Tricine SDS PAGE based electrophoregram of milk samples subjected to ion exchange chromatography. A, B, C, D, F, G samples from milk of mastitic buffalo ,C_6, C_7, C_8, C_9, C_{10} samples from control healthy milk of buffalo. Mu: ultra colour marker for low molecular range (M.W.1060-26600KDa).

low molecular peptides and characterization. Once characterized fully, these peptides could be useful for modulating the immune response using the recombinant DNA technology and this could be an alternative to antibiotic treatment for mastitis in near future.

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