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A NOVEL ANTIBACTERIAL PEPTIDE FROM WHEY-BASED FERMENTED PRODUCT OF *LACTOBACILLUS INGLUVIEI*: VALORIZATION OF WHEY WASTE

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ABSTRACT ■ A vast amount of unutilized whey of cheese industry is valorized through bacterial fermentation and generates potent peptide against multidrug resistance bacteria. An antimicrobial peptide of 4892.26 Da was produced by a *Lactobacillus ingluviei* ADK10 (GenBank- JQ395039) fermentation of whey. The effectiveness of this peptide against hospital isolate multidrug resistant *Vibrio cholerae* was investigated; the MIC and MBC (LD50) values were 35 and 70μg/ml respectively. Further, safety measurements like Hemolytic assay and In vitro cytotoxicity assay was performed. Tested peptide displayed a very negligible membrane-damaging of red blood cells and no structural changes were found in live Human colon adeno-carcinoma grade II cell. In Scanning electron microscopy, it was proved that the peptide specificity was targeted towards the bacterial cell wall damage and aggregate bacterial cell together. Hence, this reported whey fermented peptide can be placed as an effective therapeutic agent for *Vibrio cholerae* infections and become a process for utilization of waste whey.

Key words: Antimicrobial; Multidrug resistance; Lactobacillus ingluviei; Vibrio cholerae; Peptides

INTRODUCTION

A total of 90.5 billion pounds of whey was estimated to have been generated as a byproduct of cheese production in 2006. Treating whey for BOD reduction before discharging it is costly. Average volume charge was $2.50 per 1,000 gallons of sewage discharged. In addition, some jurisdictions also had surcharges on COD and other pollutants. These various charges highlight the high cost of surplus whey disposal. Making whey products reduces the surplus whey volume, saves on the cost of disposing of whey, and has the prospect of breaking even or making profit in whey plant operations (Belem & Lee 1998; Cudic et al. 2002).Thus, it is important for the industry to find new ways to use more whey.

Antimicrobial peptides (AMPs) are the most important and effective precursors to fight against the increasing emergence of drug resistant bacteria (Hancock & Lehrer 1998). AMPs have broad-spectrum activity against a wide range of micro-organisms including viruses, Gram-positive and Gram-negative bacteria, protozoa, yeasts and fungi (Larrick & Wright 1996; Peschel 2001). 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,

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stored as propeptides or mature C-terminal peptides that are only released upon proteolysis (Gobbetti et al. 2002) The antimicrobial peptides of casein origin were identified by Gobbetti et al. (2002) who isolated antibacterial glycopeptides, known as casecidins.

This study was intended to control the *V. cholerae* because it is the most common water born pathogen species responsible for cholera causing significant morbidity, mortality, and incurring healthcare costs worldwide (Smith et al. 1999). Certain antibiotics are critical to human medicine because drug available to treat human infections caused by multi drug resistant pathogens or because alternative therapies are less effective or are associated with increased side effects. In vitro transfer of plasmids carrying resistance determinants was recorded from fish pathogen to human pathogens including *Vibrio cholerae* (Bauer et al. 1966).

In this context, this experiment describes the isolation and characterization of anti-cholerae peptide from fermented whey by *L. ingluviei* ADK10 which has significant with antibacterial activity against *V. cholerae*, a clinically isolated resistant organism against a large number of conventionally used antibiotics; valorization of large amount of whey into essential antibacterial substance.

MATERIALS AND METHODS

Sample preparation

Whey sample was collected from local cow milk processing shops of Medinipure, West Bengal, India. Whey Sample was pasteurized at 67 °C for 30 min, cooled at fermentation temperature and immediately inoculated by adding 2% (v/v) of 18 h-old testing cultures grown in Man Rogosa Sharpes (MRS) broth and incubate at 37 ?C for 24 hour. The culture suspension (100 ml) was centrifuged for 10 min at 13,000 rpm to precipitate the residual debris. The supernatant was then passed through 5 kDa Biomax polyethersulfone (PES) cut-off membrane using Amicon Stirred Ultrafiltration Cells (MA, USA). The filtrate was then lyophilized and redissolved in 1ml of 5% (v/v) acetonitrile solution containing 0.01% (v/v) trifluoroacetic acid (TFA). Two type of control were prepared using the culture medium without bacteria and in MRS culture media with the bacteria.

Peptide purification

Resuspended sample was fractionated by reverse phase-HPLC (Agilent 1100 series, USA) with a ZORBAX 300 SB C18 column (4.6mm×150mm, particle size 5μm). The sterile Milli-Q water with 0.1% TFA (A) and 80% acetonitrile with 0.1% TFA (B) were used as mobile phase. The system was operated at 1ml/min flow rate with linear gradient of solvent B (upto 60%) for 50 min and the detection was monitored at 215 nm in a diode array detector. Selected peaks of the HPLC chromatogram were collected using a fraction collector (GILSON, France) coupled with the system. Fractions were concentrated by Speed-Vac and each fraction was resuspended in same solvent composition where they were eluted and tested for antimicrobial activity against clinically isolated *V. cholerae*,.

Antimicrobial assay

V. cholerae, strain was collected from NRS medical college and Hospital, Kolkata, India. Strain was cultured from a -70 ˚C stock when required and grown to mid-logarithmic phase in nutrient broth media (HiMedia, India) at 37 ?C with shaking. The zone of inhibition with isolated peptide and respective antibiotics was determined following the Kirby–Bauer disk susceptibility test (Bauer et al. 1966). All disks [5mm diameter, HiMedia, India] contained different concentrations (μg) of the respective antibiotics and 50μl of active

peptide (70μg/ml).

MIC value of the active peptide fraction was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines, 2007. The concentration of peptide used for the assay ranged from 4 to 257 μg/ml. MIC value was determined by the microtiter plate dilution method (Wang et al. 2004). Minimum bactericidal concentration (MBC) was determined by colony forming unit (CFU) of each concentration following dilution plating method (Giacometti et al. 2000; Kwakman et al. 2006). Determination of all values was carried out in triplicate and repeated at least four times for accuracy.

Non-reducing gel-electrophoresis and in-gel antibacterial test

The molecular weight of the lyophilized peptide fraction were analyzed by PAGE (12.5 %) electrophoresis (Laemmli et al. 1970). Peptides were visualized by staining the gel in one side with Coomassie brilliant blue R-250 (Mark, India). The other portion of the gel was used to perform in-gel antibacterial analysis according to the method of Palacios et al. (1999). In brief, after electrophoresis one side of the gel was cut-off and put into a petri plate and the make a thin larger over it with sterile molten (~40ºC) nutrient agar, containing *V. cholerae*, (1%). Then incubate the plate at 37°C for overnight. The zone of growth inhibition of the shaded by each band of protein was observed.

MALDI-TOF mass spectrometry

The molecular mass of the HPLC purified peptide fraction (fr-3) was also determined by Voyager DE ProTM mass spectrometer equipped with 337 nm N2 laser (Applied Biosystem, USA) (Mandal et al.2009).

Field emission-scanning electron microscopy (FE-SEM)

Peptide treated bacterial cells were harvested and washed with PBS for several times. The cells are then resuspended in same saline buffer and 5–10μl of culture was then placed onto the lysine coated glass cover slip as drop caste method. Surface morphology was studied by using a field emission scanning electron microscope (FESEM) of Carl-Zeiss, model SUPRATM 40, with an accelerated voltage 15–20 kV.

Hemolytic assay

Hemocompatibility of the purified peptide fraction was studied using standard protocol of Mandal et al (2009). Hemolytic effect of each treatment was expressed as percentage of cell lysis relative to the +ve control cells (% control) using the following formula: [(Abs570 of samples)/ (Abs570 of +ve control cells)]×100, where absorbance is abbreviated to Abs.

In vitro cytotoxicity assay

Cytotoxic effect of the purified antimicrobial peptides to HT-29 cells (Human colon adenocarcinoma grade II cell line) was examined as described previously (Chauviere et al. 1992).

Statistical analysis

The experiments were performed thrice, in triplicate each time. The standard deviation for each experimental result was calculated using Microsoft Excel. The standard deviation for each value was =5%.

RESULTS

Peptide isolation and characterization

Peptides less than 5 kDa were purified from fermented whey by membrane filtration and followed by reversed phase-HPLC. Fig. 1a shows HPLC chromatogram of isolated peptides. Nine peaks were detected in HPLC chromatogram with a major peak at 20 min which eluted by approximately 51% of solvent B. The antibacterial activity of each fraction with equal concentration was tested against *V. cholerae* by disk diffusion method. Only,

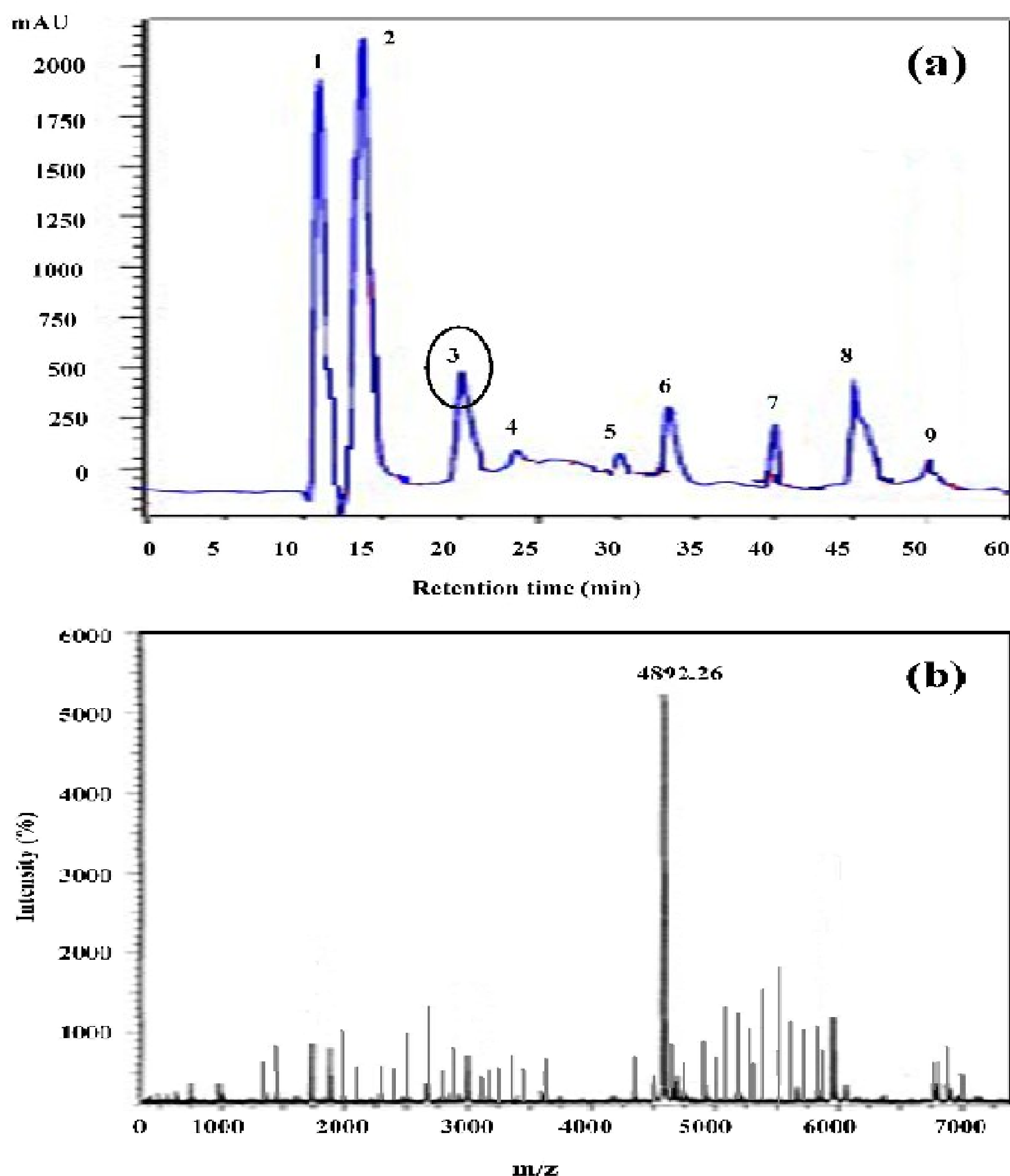

Fig. 1: (a) Reversed-phase HPLC chromatogram profile (ZORBAX 300 SB C18 column; 4.6mm×150mm, particle size 5m) of <3 kDa fraction from fermented feather hydrolysate. The sterile Milli-Q water with 0.1% TFA (solvent A) and 80% acetonitrile with 0.1% TFA (solvent B) were used as mobile phase. Diagonal line indicates a linear gradient of solvent B (0–60%). (b) MALDI TOF mass spectrum of peptide. Spectrum was acquired from Fr.3. All the spectra were recorded in the linear, positive ion mode with an accelerating voltage 20 kV and average 100 laser shots. The reproducibility of the spectra was checked several times.

fraction 3 (Fr.3) showed potent inhibitory activity against *V. cholera* and list of different tested antibiotics against used *V. cholerae* are shown in table-1 (antibiotic susceptible assay).

Gel electrophoresis and in-gel antibacterial effect study

Coomassie blue stained polyacrylamide gel of the fermented hydrolysate preparations showed a series of molecular masses of approximately 3 to 66 kDa (Fig.2), but no significant low molecular weight protein bands were appeared in the whey media without bacteria and in normal. It's indicating the protein bands appeared in the lower part of the gel is coming from the degraded whey protein residue by bacterial enzyme. It was probably the polypeptides.

Mass spectrometric study and peptide sequencing

MALDI TOF-MS analysis of fraction 3 showed that the mono isotopic molecular mass of the peptide was 4892.26 Da (Fig. 1b). The small fragment of the peptide was derived from the degraded whey protein. The sharply signal peak having molecular masses of 4892.26 Da must be derived from the degradative products of whey protein, probably

Table 1. Determination of individual zone diameter values recorded for *V. cholerae* strains (pathogenic, hospital isolate). Disks (5 mm) with individual antibiotic and peptide were used. Data are the mean of triplicate.

Antibiotics	Zone of inhibition (diameter, mm)
Gentamycin	23 (sensitive)
Kanamycin	30 (sensitive)
Amekacin	30 (sensitive)
Azithromycin	28 (sensitive)
oflaxacin	28 (sensitive)
Tetracycline	10 (sensitive)
Erythromycin	7 (resistant)
Penicillin	7 (resistant)
Choramplenical	6 (resistant)
Novobiocin	5 (resistant)
Fermented whey protein (peptides, 70 μg/ml)	**21** (sensitive)
Cephalothin	resistant
Clindamycin	resistant
Co-trimoxazole	resistant
Vancomycin	resistant
Ampicillin	resistant
Oxacillin	resistant
Linezolid	resistant
Claithromycin	resistant
Methecillin	17 (sensitive)
Amexyclan	18 (sensitive)

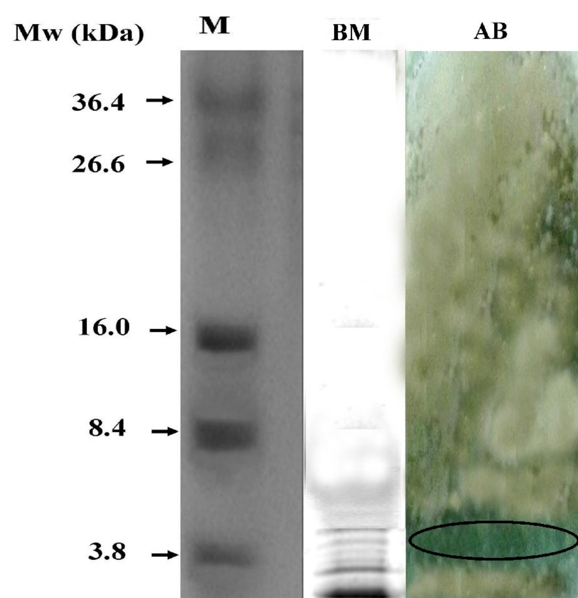

Fig. 2: Detection of antibacterial peptide (Li-antibac-3) in native PAGE using coomassie blue stain. Lane (M) = molecular weight markers, lane (BM)= whey after bacterial fermentation, lane Lane (AB) indicates the bioassay of peptide (Li-antibac-3) after native PAGE using *V.cholerae* as sensitive strain. The position of the Li-antibac-3 (MW 4.892) showing inhibitory activity was indicated in an oval box.

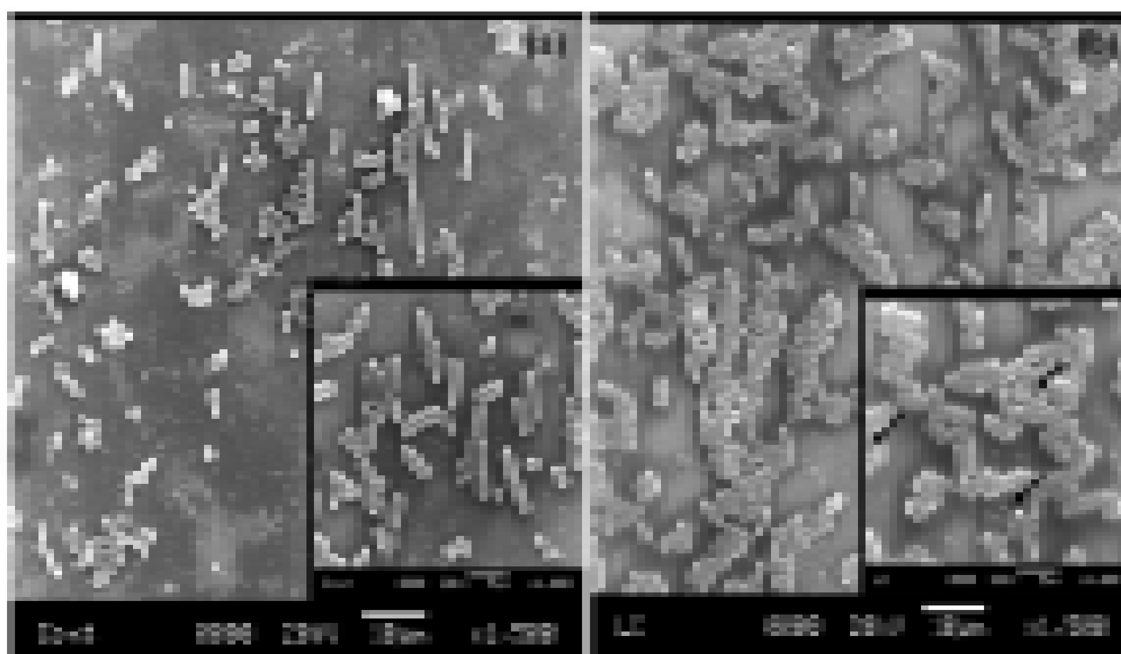

Fig. 3: Scanning electron micrographs of fraction 3 treated *V.cholerae*. Bacterial cells were incubated at 37 ?C for 1 h with peptide (Fig-b) (4μg/ml) and without peptide (control, Fig-a). The images show membrane blabbing formation with leakage of cellular contents highlighted with white arrowheads.

polypeptide (oligopeptides 20<40-50 polypeptide> protein).

Antibacterial activity

The antibiotic susceptibility assay showed that the *V.cholerae* strain was resistant to many antibiotics listed in Table 1. The MIC and MBC (LD50) values of the peptide were 35 and 70 μg/ml, respectively. The values were few folds lower than several earlier reported peptides against *V.cholerae.* Further, the interaction of the bacterial membrane and peptide was observed using FE-SEM (Fig. 3). It was clearly seen that the bacterial cell envelop was ruptured (Fig. 3b) in compared to the control bacteria (Fig. 3a).

Evaluation of red blood cell lysis

The membrane-damaging property of Li-antibac-3 peptide was tested against the mice RBC by quantifying the released hemoglobin. After 30 min and 60 min of incubation of fresh RBC with Li-antibac-3 (240μg/mL), it displayed a very negligible membrane-damaging (Fig. 4a). No significant difference in hemolysis could be detected between Li-antibac-3 and (-ve) control after 30 min of incubation, whereas extending the incubation time 60 min did show the 5% hemolysis by the peptide in respect to (+) ve control(Fig. 4b). It revealed that Li-antibac-3 peptide has no adverse effect on red blood cells at concentration of 240μg /mL.

Cell toxicity study

The toxicity of the peptide fraction 3 to HT-29 cells was examined by phase contrast microscopy and no adverse toxic effect of this peptide was found (Fig.5). No structural changes were found in live cell with out peptide (control) and cell incubated with active peptide fraction 3.

DISCUSSIONS

Several hundreds of antimicrobial peptides (AMPs) have been isolated and identified from a variety of organisms. Their mode of action includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components have been well documented. In this study, Li-antibac-3, one novel

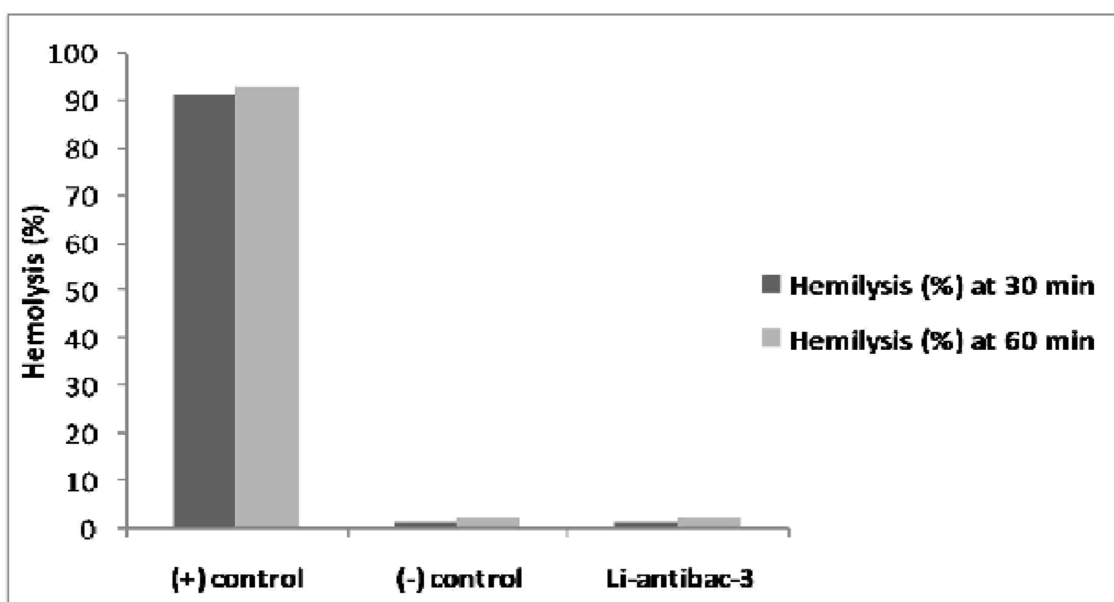

Fig. 4: Hemolytic assay of Li-antibac-3 (128 μg/ml) for 30 min and 60 min incubation at 37 °C. Data are the mean of triplicates ± S.D.

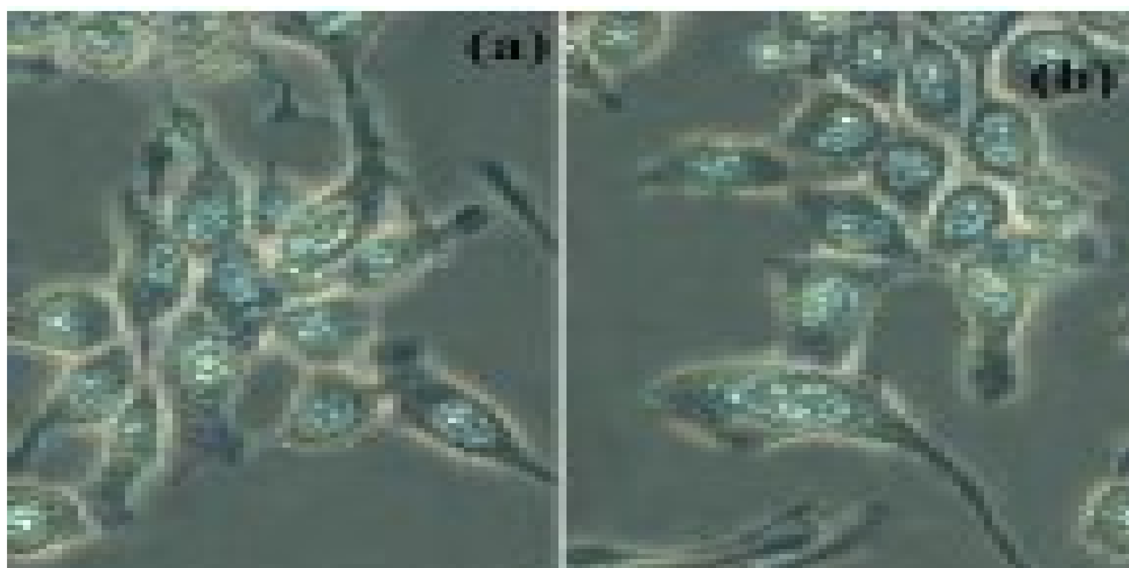

Fig. 5: Cytotoxic effect of Li-antibac-3 on HT-29 cell cultures observed under phase contrast microscope (40X) without staining. a-Blank HT-29 cell line, b- peptide treated with HT-29 cell line

antimicrobial peptide with 4892.26 Da molecular weight was isolated and identified from fermented whey with *Lactobacillus ingluviei* ADK10. Li-antibac-3 showed potent antagonistic activity against *V. cholerae*, a multidrug resistant clinical isolate. Several multidrug resistant *V.cholerae* have been described previously (Boyce et al. 1999, Shaw et al. 1970) reported that several *S. epidermidis* strains were resistant to chloramphenicol and the isolated peptide created nonspecific pores on the bacterial cell envelop and the protoplasmic content released through the perforated envelops. Hydrophobicity and chain flexibility is important factor of antimicrobial peptide (AMP) for the antibacterial activity (Friedrich et al. 2000, Zelezetsky et al. 2005). Li-antibac-3 showed acidic properties and having a significant hydrophobicity suggesting that the main cause for bacteriocidal activity. Hydrophobic interaction probably is the initial attraction between AMPs and outer bacterial envelope. SEM images revealed the interaction between peptide and *V. cholerae* membrane. A significant damage was also observed in cell membrane and membrane permeability of the cell was increased resulting growth inhibition of *V. cholerae*. Additionally, No adverse effect to epithelial cells and RBC cell has been considered as one of the selection criteria for human applicability thus in the present investigation, the peptide showed cell compatibility and can be applicable to animal as well as human being according to the report of Eliassen et al (2006).

Thus we can utilize waste product of cheese: whey into substances related to mankind. Thus it may be a promising candidate as clinically useful antimicrobial drug for treatment of antibiotic resistant cholera and diarrheal infections.

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