



Original Research Article

Effect of bioactive peptides derived from fermented whey based drink against food borne pathogens

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ABSTRACT

Keywords

Anti-pathogenic;
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The present research was carried out to examine the antipathogenic potential of bioactive peptides fractions derived from whey based fermented probiotic beverage. The fermented whey beverage prepared with proteolytic probiotic *Lactobacillus acidophilus* NCDC195. Peptides of molecular weight of 10KDa and 5KDa were obtained through step wise ultra-filtration using molecular weight cut-off (MWCO) membranes and anti-pathogenic potential of these fractions was tested. Pathogenic cultures viz. *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae* and *Escherichia coli* were taken in the study. For assessing the anti-pathogenic potential of 10Kda and 5KDa permeates against deadly food borne pathogens agar well diffusion assay was performed. The 10KDa and 5 KDa peptides rich permeate were active against both Gram-negative and Gram-positive pathogens as zone of inhibition ranged between 17 to 23 mm. Based on results it can be concluded that bioactive peptides can be used as preventive measure against food borne diseases. These biological properties may play an important role in the development of therapeutic foods that mitigate the effects of diseases.

Introduction

The increasing incidence of deadly pathogens i.e., *Listeria monocytogenes*, *Staphylococcus aureus* and cytotoxin producing *Escherichia coli* O157:H7 in foods, combined with new consumer concerns placing limits on the use of traditional antimicrobial chemical agents and salt, are pressuring food manufacturers to develop new preservatives (Mathieu et al,

2013). Milk proteins are precursors of many different biologically active peptides including antimicrobial ones. To show any antimicrobial activity, those milk-derived peptides have to be first released from their parent molecules. This can be provided either fermentation with selected proteolytic lactic acid bacteria (LAB) (Hayes et al, 2006) or by hydrolysis of the parent molecules (caseins and whey proteins) by

digestive proteases (Clare and Swaisgood, 2000). In recent years, whey derived proteins have been recognized as a valuable source of antimicrobials, demonstrating various health benefits in humans. The content of these proteins may vary between different species (Miranda et al 2004; Uniacke-Lowe et al, 2010). Releases of biologically active milk peptides occur usually during digestion of milk proteins in the gut, during enzymatic hydrolysis, chemical processes and fermentation.

Lactic starter cultures contain several proteolytic enzymes which are responsible for breakdown of milk proteins into peptides and amino acids during fermentation. Many probiotic lactobacillus strains possess good proteolytic activity i.e., *Lactobacillus acidophilus*. It has been reported that the bacteria release antimicrobial peptides from milk source (Ruixiang et al, 2007). These peptides decrease risk factor of some disease and/or to prevent disease development result of their hormone-like properties (Kitts and Weiler, 2003). Under the light of given facts the present study was carried out to study the biological activity (antipathogenic potential) of whey derived peptides against killer food borne pathogen.

Materials and Methods

Collection of material

Whey was collected from Experimental Dairy, National Dairy Research Institute, Karnal. Proteolytic probiotic culture *Lactobacillus acidophilus* NCDC 195 was originally obtained from National Collection of Dairy Culture, NDRI, Karnal, India. The standard bacterial pathogens were taken from different sources i.e., *Staphylococcus aureus* (MTCC 1144) from Microbial Type Culture Collection, Chandigarh, India and *Listeria monocytogenes* (SCOTT A3) from

the STELA research center strain collection (Quebec, QC, Canada). Other pathogens i.e., *Bacillus cereus* (NCDC-240), *Salmonella typhi* (NCDC 113), *Escherichia coli* (NCDC 135), *Enterococcus faecalis* (NCDC 115), *Shigella dysenteriae* (NCDC 107) from National Collection of Dairy Cultures, NDRI, Karnal, India. Chemicals, media, and other materials were procured from Tarsons, Himedia, Merk, Sigma etc.

Maintenance and cultivation of the cultures

Proteolytic *L. acidophilus* NCDC 195 was activated in sterile skim milk medium. The culture was maintained by sub-culturing fortnightly in skim milk using 1% inoculum and 24 hrs of incubation at 37°C and stored after activation at 4°C between transfers. The culture was sub-cultured twice in MRS broth (HiMedia) from skim milk before use. Pathogenic cultures were activated in BHI broth (HiMedia) before use.

Analysis of prepared probiotic fermented whey beverage

Probiotic fermented whey beverage was prepared using method previously optimized for its preparation (data not shown) by inoculating with 1.5 % culture. Fermentation was done at 37°C for 24 hrs. Total soluble solids were measured using a hand refractometer of 0-32°B (ERMA make). The pH of the beverages was determined using the digital pH meter (Orion pH meter). Titratable acidity was determined according to the AOAC (Williams, 1984) method. Protein estimation was done by Micro-Kjeldahl method (Williams, 1984). Viable counts in the samples were determined according to A.P.H.A. (Vanderzant et al, 1992) procedure using lactic agar (Elliker et al 1956). Peptides released in probiotic fermented whey beverage through

proteolytic potential of probiotic culture (*Lactobacillus acidophilus* NCDC 195) were quantified using the o-phthalaldehyde (OPA) method (Benson et al, 1975).

Extraction of bioactive peptide rich 10 KDa and 5 KDa fractions from probiotic fermented whey beverage

The probiotic fermented whey beverage was step wise filtered to obtained peptide rich fraction. Firstly, the probiotic fermented whey beverage supernatant was obtained by centrifugation at 7000 rpm for 10 minutes in refrigerated centrifuge. The supernatant was filtered with 0.45 μ m syringe filters and the obtained filtrate was filtered with 0.22 μ m syringe filters. 10KDa and 5KDa ultra filtrate bioactive peptide fractions were obtained by passing supernatant through 10 KDa and 5KDa MWCO membranes (Vivaspin) and these fractions were assayed for antipathogenic potential.

Antipathogenic potential of 5 KDa and 10KDa fractions

The fermented whey drinks peptide fractions of 10 KDa, and 5KDa were screened for their antimicrobial activity against various indicator organisms using agar well diffusion assay as per the method of Perez et al (1990) with some modifications. The indicator organisms were grown for 16-18 h at their optimum temperatures in BHI broth. 100 μ l of the culture was transferred to 5ml of Brain Heart Infusion broth and incubated for four hours so that all cells should be in log phase. 50 μ l of this culture which contained 10⁶ cfu/ml was transferred to soft agar and poured onto the base plates already prepared.

The plates were refrigerated at 5° C for an hour before wells were cut into these agar plates. The bottom of the wells was sealed

and 50 μ l of permeate was placed into each well.

The plates were stored at 4° C for 2h to permit radial diffusion of the permeate and incubated at 37°C for 24 h and subsequently examined for zone of inhibition surrounding each well. A clear zone of 1 mm or greater extending laterally from the edge of well was considered positive inhibition.

Statistical analysis

Statistical procedures as described by Snedecor and Cochran (1977) were used to analyze the data for the interpretation of results. Mean, standard deviation and analysis of variance (ANOVA) were used to describe the results.

Result and Discussion

L. acidophilus is one of the most popular species of lactic acid bacteria. In the present study probiotic proteolytic *L. acidophilus* strain NCDC 195 was taken.

Analysis of probiotic fermented whey beverage

As shown in results the log count of the prepared probiotic fermented whey beverage were 8.59 cfu/ml, the values obtained for pH, titratable acidity and total soluble solids were 4.02 \pm 0.02, 1.02 \pm 0.01 and 20.83 \pm 0.03 respectively. The peptide contents were 469.85 \pm 0.12 μ g/ml.

Bhardwaj and Singh (2013) reported that peptide of whey supernatant was observed higher at 37°C while at higher temperatures peptide contents get reduced this is because of production of more peptidases at higher temperature i.e. 40°C and 44°C.

Antipathogenic potential of bioactive peptide rich 5KDa and 10KDa fractions of fermented whey beverage

Before obtaining the supernatant for ultra-filtration pH of the sample obtained from probiotic fermented whey beverage was adjusted at 6.0 thus the effect of lactic acid was eliminated. The bioactive peptide rich 5KDa and 10KDa fractions obtained from probiotic fermented whey beverage had shown good antipathogenic potential. These fractions had different activity towards a specific food born pathogen. It is clear from the Table 2 that peptides derived through the *L. acidophilus* strain NCDC 195 had potential to inhibit deadly food borne pathogens.

The 5KDa fraction of whey derived peptides was active against both Gram positive (*B.cereus*, *L. monocytogenes*, *Staph. aureus* and *E. faecalis*, with zone of inhibition 21 mm, 19 mm, 19 mm, and 21 mm, respectively) and Gram negative pathogens *E.coli*, *S.typhi*, *Sh. dysenteriae* with zone of inhibition 18mm, 18 mm and 17 mm, respectively (Fig 1). Same was with 10KDa fraction, as it was also active against both types of pathogens (*E. faecalis*, *B.cereus*, *L. monocytogenes*, *Staph. aureus* with zone of inhibition 29 mm, 20 mm, 23 mm and 18 mm, respectively and Gram negative pathogens *E.coli*, *S.typhi*, *Sh. dysenteriae* with zone of inhibition, 17mm, 17mm and 16 mm respectively (Fig 2). It was very clear from the results that Gram positive pathogens are very sensitive as zone of inhibition ranged between 19mm to 23mm. Gram negative pathogens were also showing sensitivity but the range of zone of inhibition is narrower as compared to Gram negative pathogens. The most sensitive pathogen was *L. monocytogenes* (SCOTT A3) with zone of clearance 23mm and least sensitive pathogen was *Sh. dysenteriae* (NCDC 107)

with 15mm zone of inhibition. Similar kind of results were obtained by Véronique et al (2013) that whey protein derived peptides were more active against pathogenic Gram positive bacteria (*L. monocytogenes* Scott A3 and *S. aureus* ATCC 25923), but less effective against Gram negative bacteria (*E. coli* O157:H7 ATCC 35150).

Antibacterial peptides from milk and whey proteins have been reported during the last 20 years with clear inhibitory effects on various strains of *E. coli*, *L. monocytogenes*, *B. cereus* and other micro-organisms (Tomita et al, 1994; Recio and Visser, 2000; Malkoski 2001; Haque and Chand, 2008).

It has been reported that the peptide rich fraction obtained from whey had a much stronger antibacterial effect than the single peptides. In the same paper it was reported that neither the peptides nor the digested whey had any antimicrobial effect on the probiotic strain LGG; it seemed rather to be activated by the whey hydrolysate (Hilde et al, 2011).

According to the literature it is clear that microbial enzymes possess the antimicrobial properties and based on data it is hypothesized that the feeding of these natural proteins results in the production of anti microbial bioactive peptides, which function as effecting antibiotics via the direct antimicrobial activity of the peptides (Bhardwaj and Singh, 2013).

The present study revealed the anti-pathogenic potential of paneer whey derived bioactive peptides. It can be concluded from obtained results that Gram positive pathogens are more sensitive from bioactive peptides derived from whey as compare to Gram negative pathogens. This type of therapeutic beverages can be used as preventive measure against food borne

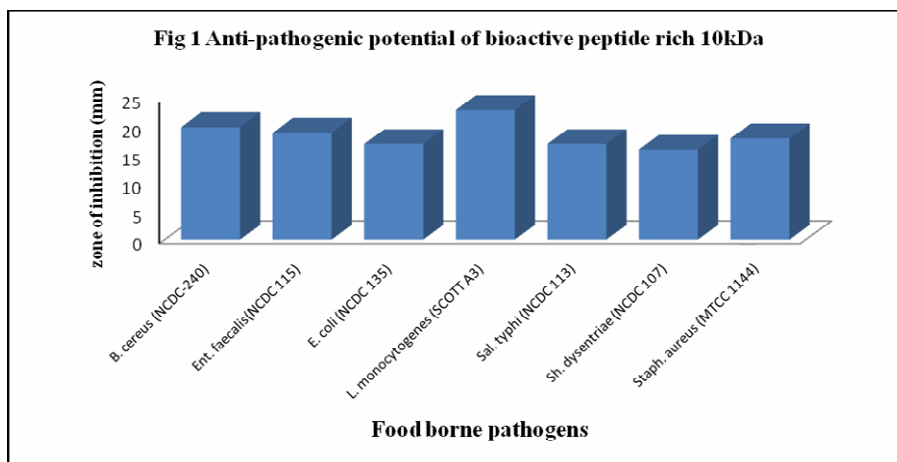
diseases. The biological properties of whey derived peptides may play an important role in the development of therapeutic foods that

mitigate the effects of killer food borne pathogen in human system.

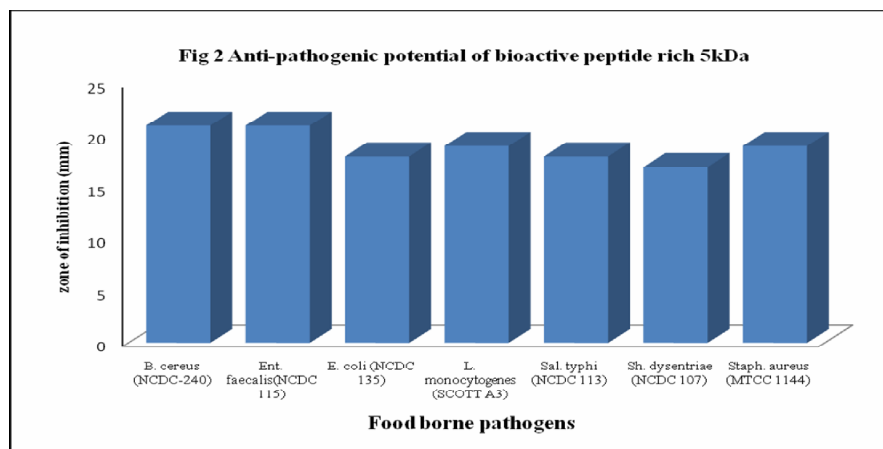
Table.1 Chemical and microbiological analysis of probiotic whey based fermented drinks

S.No.	Parameters	Probiotic whey based drink
1.	pH	4.02±0.02
2.	Acidity(%LA)	1.02±0.01
3.	Lactic Count (Log10 Cfu/ml)	8.59±0.17
4.	Tss (%Brix)	20.83±0.03
5.	Peptide Content (µg/ml)	469.85±0.12

Data are presented as Mean ±SD (n=3)



Data are presented as Mean ±SD (n=3) (zone of inhibition diameter in mm)



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