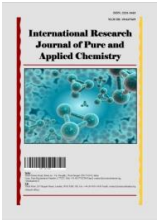

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Studies on Immunomodulatory Effect of Casein Phospho Peptide Isolated from Cultured Dairy Product

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Authors' contributions

This work was carried out in collaboration among all authors. Author IM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors VK and BD managed the analyses of the study. Author RS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Bioactive peptides have been defined as specific protein fragments that have an impact on body functions or conditions and may ultimately influence health. Fermented milk is a dairy product which has abundance of bioactive peptides. In this study, Casein Phospho peptide (CPP) was isolated by enzymatic hydrolysis of fermented milk using trypsin. The molecular weight of the Casein Phospho peptide was 3.5 KDa. The anti-bacterial activity of Casein Phospho peptide was determined using

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four pathogens such as, *Escherichia coli, Bacillus cereus, Staphylococcus aureus* and *Salmonella enterica.* Casein phospho peptide formed a zone of inhibition against the pathogens. The bioactive peptides were characterized using Fourier Transform Infra-Red Spectroscopy (FT-IR), Casein Phospho peptide had aliphatic amine, acetyl amino I and acetyl amino II functional groups. The HPLC analysis of Casein Phospho peptide revealed that the major amino acid present was L-Glutamic acid and the amino acid present in lesser concentration was Leucine. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from human blood and the cells were treated with Casein Phospho peptide to assess the immunomodulatory effect.
Casein Phospho peptide was able to produce a higher concentration of IL-10 anti-inflammatory cytokines when treated with PBMCs.

Keywords: Fermented milk; CPP; FT-IR; HPLC; anti-bacterial activity; immunomodulatory activity.

1. INTRODUCTION

Milk fermented by specific Lactic acid bacteria releases numerous natural bioactive compounds that provide an excellent pool of molecules for the production of nutraceuticals, functional foods, and food additives [1,2]. The amino acids released by the bacteria accumulate in the milk and affect the nutritional potential and biological value of the fermented product. Amino acids may not directly contribute to the flavor and aroma of fermented milk. However, they act as precursors for several reactions that produce carbonyl compounds [1].

Lactic acid bacteria are fastidious microorganisms and require an exogenous source of amino acids or peptides for optimum growth. The growth of the starter bacteria in milk is dependent on their proteolytic systems to hydrolyze caseins. Milk and milk products contain bioactive components that have been shown to increase the activity of the immune system. Evidence suggests that these peptides assist in the proliferation of lymphocytes, the functioning of natural killer cells, the synthesis of antibodies, and the production of cytokines [2,3].

The bioactive peptides are liberated from the native protein *in vivo* by digestive proteases or by enzymatic hydrolysis secreted by microorganisms during fermentation [4]. As a result of proteolysis, proteins are broken into bioactive peptides. Bioactive peptides can be released by enzymes from food precursor proteins during processing (ripening, fermentation, heating), storage, and in vitro proteolysis. Many microorganisms found in dairy starter cultures are highly proteolytic [5].

The bioactive peptides or other compounds released during fermentation of milk with LAB show immunomodulatory activity. Indeed, many beneficial effects have been attributed to bioactive peptides derived from milk, including opiate activity, antimicrobial activity, antihypertension, antithrombotic activity, and immunomodulation [3,4].

Fermented milk products with lactic acid bacteria have been shown to enhance both innate and adaptive immune responses. The immunomodulatory activity of lactic acid bacteria includes the activation of the systemic and secretory immune responses through interactions between microbiota epithelial cells and immune cells [6,7].

Bioactive peptides are generated by the starter and non-starter bacteria that are used in the manufacture of fermented dairy products [8,9]. This system consists of a cell wall-bound proteinase and several distinct intracellular peptidases, including endopeptidase, aminopeptidase, tripeptidase, and dipeptidase [10].

The commercially available foods having bioactive peptides, derivatives of milk proteins, and other proteins, is very limited [11-13]. Bioactive peptides are a rapidly growing field of research that should significantly contribute to the availability of functional foods on the market shortly [14,15].

This study is aimed at studying the immunomodulatory property of the bioactive peptide present in fermented milk and characterization of the Casein Phosphopeptide was done using SDS-PAGE, FT-IR, HPLC.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Lactic acid, Sodium Hydroxide, MRS broth, MRS agar, Glucose, Sodium Chloride, Bromophenol

blue, Hydrochloric acid, Trypsin enzyme, Calcium chloride, ethanol, Ammonium sulfate, Tris-HCl, Glycerol, β-mercaptoethanol, Acrylamide-Bis acrylamide, TEMED, Coomassie brilliant blue R-250, Glacial acetic acid, Luria-Bertani agar, MRS broth and agar (Himedia), Antibiotic Kanamycin (1mg/ml), Histopaque (1077- Sigma Aldrich), DMEM.

2.1.2 Bacterial strains

Lactobacillus acidophilus (NCDO 2), *Lactobacillus delbrueckii* subsp. *bulgaricus* (NCDO 1489), *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 23235, *Bacillus cereus* (ATCC 14579) and *Salmonella enterica* (ATCC 35664).

2.2 Methods

2. 2.1 Preparation of fermented milk

Fermented milk was prepared as per the method adopted by Heller (2016). The whole milk was boiled to 80°C for 15 mins and then cooled to 42°C. Further, the whole milk was inoculated with *acidophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* at the inoculation rate of 5%. Then it was incubated at 37°C overnight. The pH and titratable acidity of the fermented milk was recorded [7].

2.2.2 Isolation of casein phosphopeptide from fermented milk

Casein suspension was prepared from fermented milk. The pH of the fermented milk was adjusted to 7 using 0.5 N NaOH. Then the trypsin (GIBCO BRL) was added to the fermented milk at an enzyme: substrate ratio of 1:100. The hydrolysis was carried out by mixing the suspension using a magnetic stirrer in a water bath at 37°C for 30 min. The pH of the solution was kept constant at 7.0 by adding 0.1 N NaOH solution. After complete hydrolysis, the mixture was removed from the water bath and the pH of casein hydrolysate was adjusted to 4.6 using 2M HCl. The mixture was centrifuged at 3000 rpm for 10 min to remove the non-phosphorylated peptides. The supernatant was collected and pH was adjusted to 7.0 using 2.0 M NaOH. Further calcium chloride was added at a 1% level to the supernatant and the mixture was allowed to sit for 1 hour at room temperature. Further ethanol 50% (V/V) was added to the above mixture. The CPP precipitate was separated by centrifugation at 8000 rpm for 10 min. The isolated CPP was further treated with 1N Lactic acid and centrifuged at 6000 rpm for 10 min. The CPP thus obtained was lyophilized [16].

2.2.3 Molecular weight determination of bioactive peptides Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS – PAGE)

The molecular weight of isolated CPP was confirmed by SDS-PAGE [17]. The 15% SDS-PAGE gel was prepared. The peptides were mixed with the sample buffer at a 1:1 ratio. Then the samples were prepared by incubating the samples in a boiling water bath for 10 mins. After incubation, the samples were snap chilled and centrifuged at 8000 rpm for 5 mins. The support was loaded into the 15% SDS-PAGE gel along with the protein molecular marker (Biorad prestained SDS-PAGE standards Broad Range #1610318). The samples were run at 100V for 2hrs. The molecular weight of the isolated CPP was determined with a help of a protein molecular weight marker after staining with coomassie brilliant blue [16].

2.2.4 Anti-bacterial activity of the bioactive peptide

The antibacterial activity of the isolated Casein Phospho Peptide was determined using *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus,* and *Salmonella enterica*. Wells was created in the agar plates (Luria Bertani agar Hi-Media) and 100 µl of all three CPP's were added along with 100 µl of antibiotic Kanamycin (1 mg/ml) as the control. The plates were incubated at 37°C for 48 hrs and then the zone of inhibition was measured. The zone of inhibition for control and test were measured in mm. The diameter of the zone of inhibition for each of the microorganisms was recorded.

2.2.5 Fourier transform infra-red spectroscopy (FT-IR) analysis of the bioactive peptides

The bioactive compounds present in the isolated peptides were analyzed by the infra-red spectra are recorded on the Fourier transform spectrophotometer in the infra-red region within the range 500-4000 Cm^{-1}.

2.2.6 High-Performance Liquid Chromatography (HPLC) analysis of the bioactive peptides

Bioactive peptides isolated were further characterized in the present study by HPLC. HPLC module Alliance 2695 with diode-array detector PDA 2996 (Waters, Milford, USA) was used. Detection was carried out at a wavelength of 205 nm. The separation was performed on a chromatographic column Poroshell 300SB-C-17, 2.1 × 75 mm, 5 µm particle size (Agilent, Santa Clara, USA). Mobile phase A consist of water/acetonitrile/trifluoroacetic acid (95:5:0.1) and mobile phase B consist of water/acetonitrile/trifluoroacetic acid (5:95:0.1). The column temperature was adjusted and set at 45°C and the injection volume was 10 µl. Data were collected and evaluated by Empower software (Waters, Milford, USA).

2.2.7 Isolation of Peripheral blood mononuclear cells (PBMCs)

Human Peripheral Blood Mononuclear cells (PBMC) were isolated from a healthy donor by the histopaque gradient method. PBMCs were isolated according to the method described by Otani et al., (2000). Briefly, peripheral blood mononuclear cells were separated from the blood samples. The 2 X 10^6 viable peripheral blood mononuclear cells per well were seeded onto 24 well flat-bottomed culture plates containing 10% growth medium (90 µl DMEM +10ml FBS + 1X antibiotic)

2.2.8 Immunomodulatory activity of Casein Phospho Peptide

The immunomodulatory activity of the peptide was performed according to the method described by Qian et al. [6]. To determine the *in vitro* immunostimulation of soluble peptides, the PBMCs were co-cultured with 100 µl of each resuspended Casein Phosphopeptide in DMEM (20 µg protein) onto 24 well flat bottom plate. Then the PBMCs were incubated at 37°C with a 5% CO_2 incubator for 72 hrs. The supernatant was collected after 72 hrs by centrifugation for 10000 rpm 10 min at 4°C and used for the assessment of cytokine production using commercial Human IL-10 ELISA kit (*Invitrogen* KHC0101).

3. RESULTS AND DISCUSSION

The milk samples were fermented by *L. acidophilus* and *L. bulgaricus* and commercial curd [17-19]. The lowest pH was observed in the case of milk fermented by commercial curd because of the presence of mixed bacterial culture which represents the utilization of lactose which in turn resulted in the increased production of lactic acid [20-22]. Milk fermented by *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* had a single organism and reduction of pH was slow when compared to commercial curd (Table 1). The lowest titratable acidity was observed in the milk fermented by Lactobacillus acidophilus and the highest titratable acidity was observed in the milk fermented by commercial curd (Table 2). The highest titratable acidity in commercial curd might be due to the presence of mixed organisms.

3.1 Isolation of Casein Phosphopeptide

The CPP was isolated from fermented milk. 1.3 g/100 ml, 1.28 g/100 ml, 1.36 g/100 ml of CPP of milk fermented by *L. acidophilus*, *L. bulgaricus,* and commercial curd respectively. The amount of CPP production was varied between the three fermented milk.

3.2 Anti-Bacterial Activity of the Bioactive Peptides

Anti-bacterial activity of the CPP was determined against pathogens like *Escherichia coli, Bacillus cereus, Staphylococcus aureus, and Salmonella enterica* (Figs. 1, 2, 3, 4). The mean zone of inhibition produced by Culture A CPP against the pathogens were 22±0.48, 23±0.33, 21±0.38, 21±0.33 respectively when compared to antibiotic Kanamycin (control) 21±0.38, 21±0.48, 21±0.58, 21±0.40. The mean zones of inhibition produced by Culture B CPP against the pathogens were 23±0.33, 21±0.36, 17±0.43, 23±0.34 respectively. The mean zones of inhibition produced by Culture C CPP against the pathogens were 22±0.47, 21±0.46, 23±0.43, 18±0.48, respectively. Statistical analysis revealed a significant difference ($P \leq 0.05$) between antibiotic control and CPP against the pathogens (Table 3).

3.3 Molecular Weight Determination of Bioactive Peptides by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS – PAGE)

The molecular weight of the CPP was confirmed by SDS-PAGE analysis and it was found to be in the range of 3.5 KDa (Fig. 5).

Table 1. pH values for control milk and fermented milk

Time (in hrs)	Control (Mean±SE)	Culture A (Mean±SE)	Culture B (Mean±SE)	Culture C (Mean±SE)
0	6.95±0.015	6.95±0.015	6.95±0.015	6.95±0.015
1	6.95±0.015	6.85±0.072	6.90±0.114	6.75±0.092
2	6.95±0.015	6.50±0.145	6.80±0.151	6.60±0.152
3	6.89±0.015	6.38±0.115	6.68±0.148	6.35±0.105
4	6.80±0.015	6.12±0.121	6.42±0.127	6.05±0.112
5	6.71±0.015	5.92±0.135	6.02±0.165	5.89±0.125
6	6.50±0.015	5.78±0.145	5.98±0.135	5.65±0.115
7	6.21±0.015	5.57±0.128	5.77±0.126	5.45±0.109

Table 2. Titratable acidity for control and fermented milk

S. No	Sample	Titratable acidity	
		Mean	Standard Error (±)
1	Control Milk	0.25	0.001
2	*Lactobacillus acidophilus*	0.79	0.035
3	*Lactobacillus bulgaricus*	0.82	0.025
4	Commercial curd	0.89	0.015

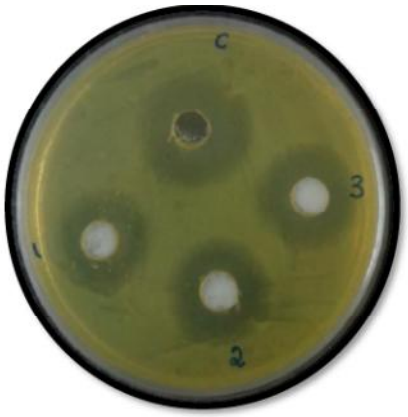

Fig. 1. Zone of Inhibition against *Escherichia coli*
1-Culture A CPP; 2- Culture B CPP; 3- Culture C CPP; C- Kanamycin control

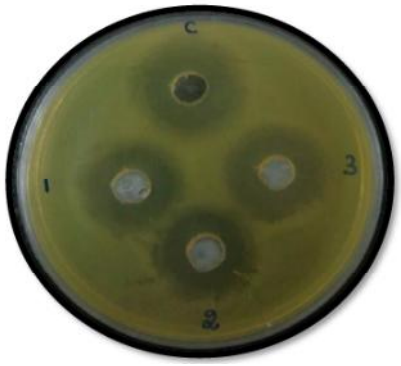

Fig. 2. Zone of inhibition against *Bacillus cereus*
1-Culture A CPP; 2- Culture B CPP; 3- Culture C CPP; C- Kanamycin control

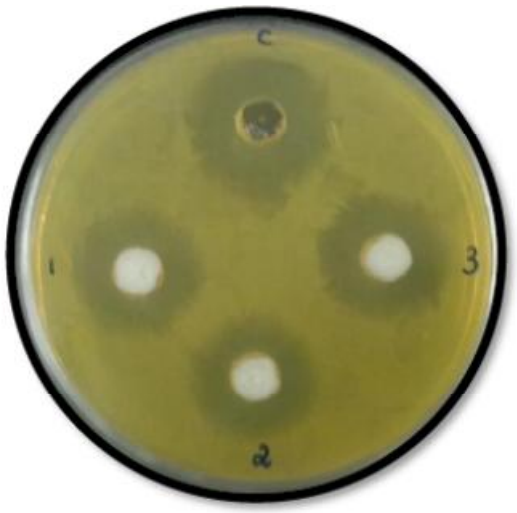

Fig. 3. Zone of Inhibition against *Staphylococcus aureus*
1-Culture A CPP; 2- Culture B CPP; 3- Culture C CPP; C- Kanamycin control

Fig. 4. Zone of Inhibition against *Salmonella enterica*
1-Culture A CPP; 2- Culture B CPP; 3- Culture C CPP; C- Kanamycin control

Table 3. Mean zone of inhibition of CPP against pathogens (Mean±SE) [@]

Pathogen	Kanamycin Control (Mean±SE)	Culture A CPP Mean±SE)	Culture B CPP (Mean±SE)	Culture C CPP (Mean±SE)
Escherichia coli	21^{a}±0.38	22^{b}±0.48	23^{c}±0.33	22^{bc}±0.47
Bacillus cereus	21^{b}±0.40	21^{b}±0.20	23^{c}±0.84	18^{a}±0.58
Staphylococcus aureus	21^{a}±0.48	23^{b}±0.75	21^{a}±0.56	21^{a}±0.76
Salmonella enterica	21^{b}±0.58	21^{b}±0.62	17^{a}±0.45	23^{c}±0.53

@Average of six trials
The mean scores with different superscripts represents that there is significant difference between columns
The mean scores with similar superscripts represents that there is no significant difference between columns

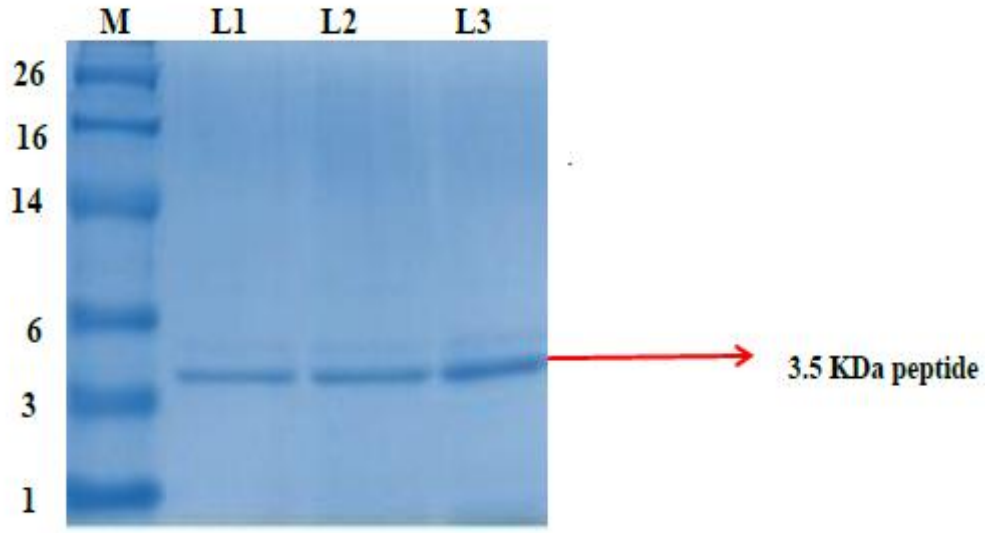

Fig. 5. 15% by SDS-PAGE gel showing Casein Phospho peptide
M- Polypeptide SDS-PAGE Marker
L 1- CPP isolated from L. acidophilus fermented milk
L2- CPP isolated from L. bulgaricus fermented milk
L3- CPP isolated from commercial curd fermented milk

3.4 Fourier Transform Infra-Red Spectroscopy (FT-IR) Analysis of the Bioactive Peptides

The bioactive compounds present in the Casein phosphopeptide was confirmed by FT-IR analysis. The FT-IR spectra of CPP isolated from milk fermented by *L. acidophilus.* was present in Chart 1. The intense peaks were obtained at 3355.96 Cm^{1}, 2981.75 Cm^{-1}, 2362.71 Cm^{-1} and 1639.40 Cm^{-1}. The peak at 3355.96 Cm^{-1} corresponds to aliphatic primary amine (N-H) stretching and the peak @ 1639.40 Cm^{-1} corresponds to electrostatic interaction between the phosphoric groups.

The FT-IR analysis of Casein phosphopeptide obtained from milk fermented using *L. bulgaricus* was illustrated in Chart 2. The broad peaks were observed at 3374.02 Cm^{-1}, 2130.21 Cm^{-1},1644.03 Cm^{-1},1454.56 Cm^{-1} reflecting primary amine (N-H), Ketene (C=C=O) and Alkene (C=C) groups respectively.

In FT-IR analysis the broad peaks were observed at 3355.96 Cm^{-1}, 2981.75 Cm^{-1}, 2362.71 Cm^{-1}, 1639.40 Cm^{-1} in the CPP isolated from milk fermented by commercial curd (Chart 3) reflecting aliphatic primary amine (N-H) stretching, Alkene (C-H), alcohol (O-H) respectively.

3.5 High-Performance Liquid Chromatography (HPLC) Analysis of the Bioactive Peptides

Casein Phospho Peptide isolated from fermented milk was subjected to High-Performance Liquid Chromatography (HPLC) analysis to study the characteristic features of fermented milk peptides. The observed peaks at 2.664 Rt, 9.126 Rt, 10.019 Rt, 10.506 Rt, 11.463 Rt, were respective to the major amino acids present in CPP isolated from milk fermented with *L. acidophilus*. Concerning the peak area of the spectrum, L. Glutamic acid content is more in the CPP isolated from milk fermented with L. acidophilus. The amino acid present least is Isoleucine (Chart 4).

The peaks observed at 2.694 Rt, 9.780 Rt, 10.019 Rt, 10.561 Rt, 11.473 Rt, were the major amino acids present in CPP isolated from milk fermented with *L. bulgaricus*. The results revealed that L. Glutamic acid content is more in the CPP isolated from milk fermented with *L. bulgaricus*. The least amino acid present in the isolated CPP was Lysine (Chart 5).

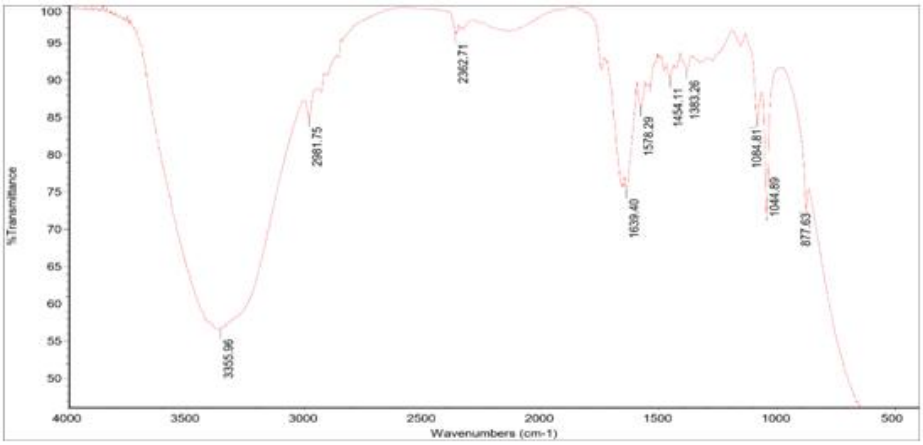

Chart 1. FT-IR spectrum for Casein Phospho peptide isolated from milk fermented using *Lactobacillus acidophilus*

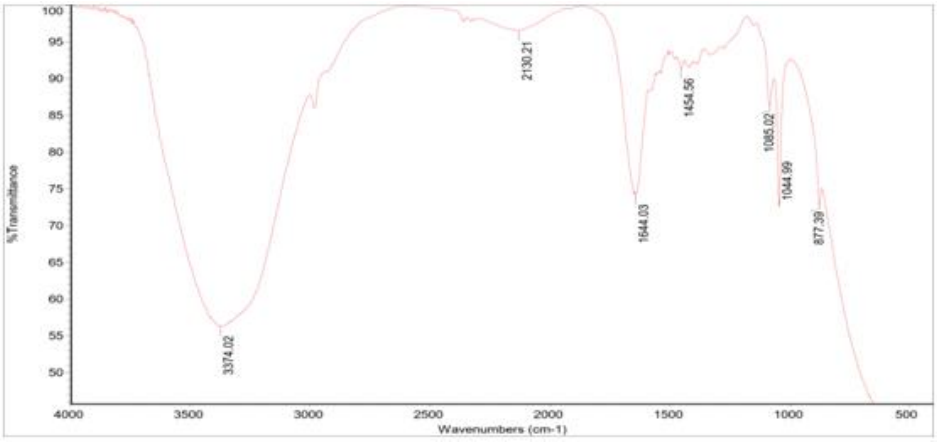

Chart 2. FT-IR spectrum for Casein Phospho peptide isolated from milk fermented using *Lactobacillus bulgaricus*

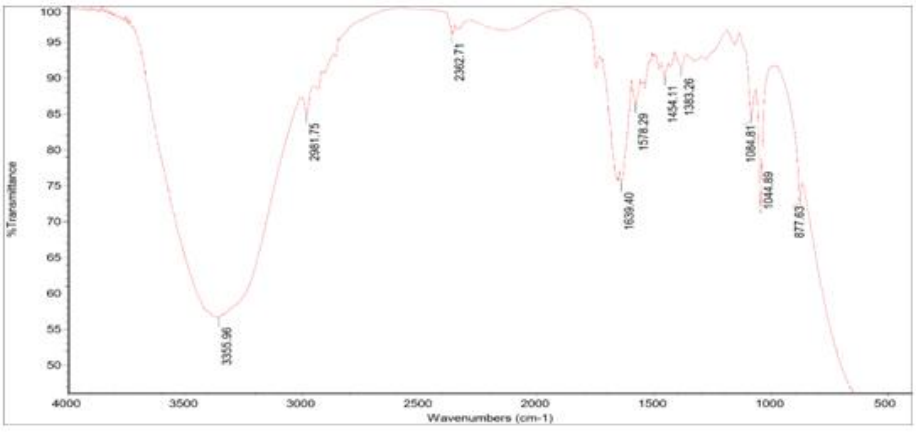

Chart 3. FT-IR spectrum for Casein Phospho peptide isolated from milk fermented using Commercial curd

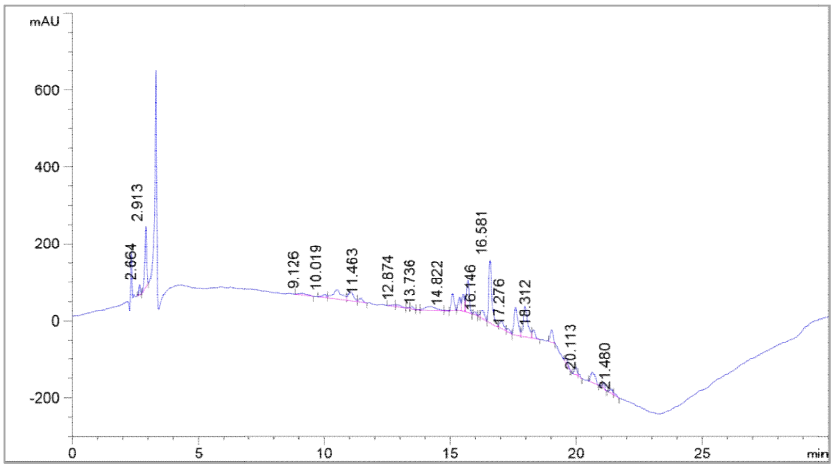

Chart 4. HPLC spectrum of Casein Phospho peptide isolated from milk fermented with *Lactobacillus acidophilus*

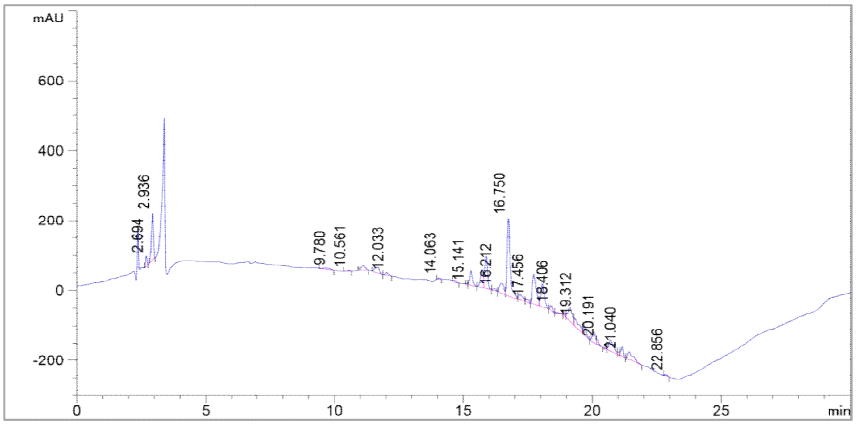

Chart 5. HPLC spectrum of Casein Phospho peptide isolated from milk fermented with *L. bulgaricus*

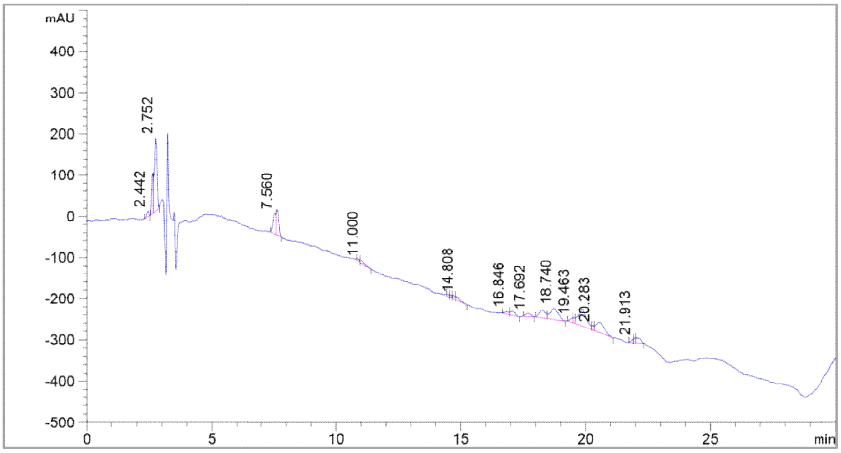

Chart 6. HPLC spectrum of Casein Phospho peptide isolated from milk fermented with Commercial curd

Fig. 6. Peripheral blood mononuclear cells at 0 hr at 20 X magnification

Fig. 7. Peripheral blood mononuclear cells at 24hrs at 20 X magnification

Fig. 8. Peripheral blood mononuclear cells at 48hrs at 20 X magnification

Fig. 9. Peripheral blood mononuclear cells at 72 hrs at 20 X magnification

Table 4. R^2 values of treated and untreated samples (Mean±SE)[@]

Cells	R^2 Values nmol/ml (Mean±SE)
Untreated cell control	0.367±0.009[a]
Culture A CPP treated	1.255±0.007[bc]
Culture B CPP treated	1.153±0.005[b]
Culture C CPP treated	1.345±0.004[c]

[@]Average of six trials

The mean scores with different superscripts represents that there is high significant difference between treatments.

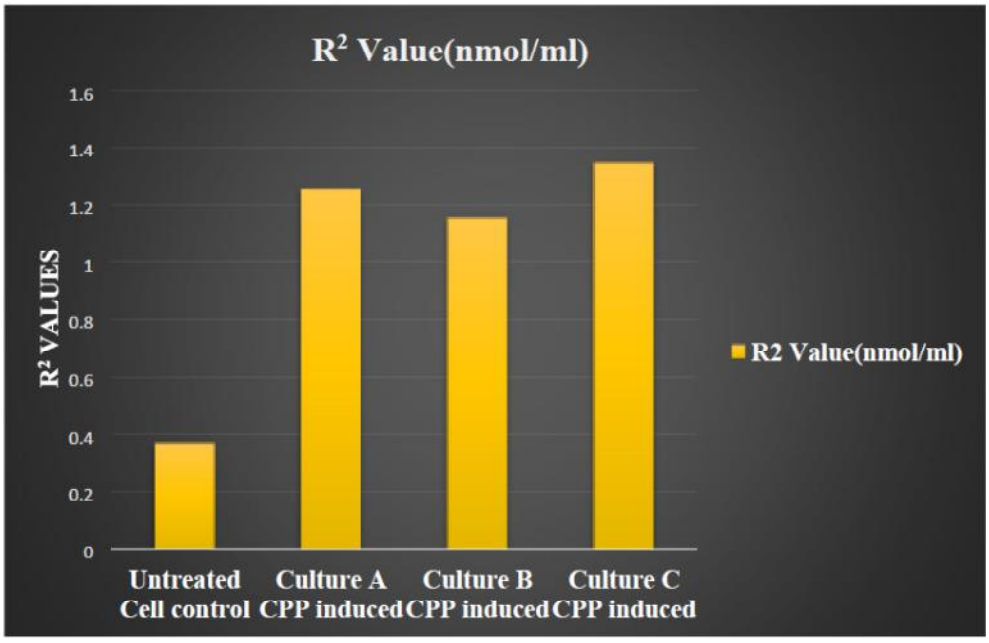

Chart 7. Cytokine production in treated and untreated cell

The peaks observed at 2.664 Rt, 9.126 Rt, 10.019 Rt, 10.506 Rt, 11.463 Rt, were the major amino acids present in CPP isolated from milk fermented with commercial curd. The results revealed that the L. Glutamic acid content is more in the CPP isolated from milk fermented with commercial curd. The amino acid present least is Lysine (Chart 6).

3.6 Immunomodulatory Activity of Casein Phospho Peptide

The Peripheral blood mononuclear cells (PBMCs) were treated with the CPPs isolated from fermented milk for cytokine production (Fig. 6, Fig. 7, Fig. 8, Fig. 9). After 72 hrs of incubation, the supernatant was separated and analyzed for cytokine production using a commercial Human IL-10 ELISA kit.

The casein phosphopeptide derived from fermented milk by *L. acidophilus, L. bulgaricus* and commercial curd stimulated the production of anti-inflammatory cytokines (IL-10) in varying concentrations compared with untreated PBMCs (Table 4 and Chart 7).

4. CONCLUSIONS

The bioactive peptide casein phosphopeptide was isolated from milk fermented using *L. acidophilus, L. bulgaricus,* and commercial curd. The molecular weight of the CPP was 3.5 KDa. FT-IR analysis showed that the functional groups present in the CPP and HPLC were done to identify the amino acids. CPP also had immunomodulatory activity, producing IL-10 cytokine. The CPP has already been added to toothpaste commercially to improve calcium absorption. Further, the CPP can be added to fruit smoothies and other beverages to boost the immune system.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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