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RESEARCH ARTICLE

ISOLATION CHARACTERIZATION AND COMPARITIVE ANALYSIS OF CASE IN OPHOSPHOPEPTIDES FROM DIFFERENT MILK SAMPLES

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A B S T R A C T

Bioactive peptides obtained from fermented milk have a varied composition and have a wide array of uses. This study was undertaken to isolate CPP from different unpasteurized milk samples which were fermented using different strains of *Lactic acid bacteria* (LAB) and it was then characterised using appropriate techniques. The two parameters of titrable acidity and pH were standardized before commencing the studies.The casein, total protein and calcium content of CPP were quantified. The casein content was determined by the rapid Walker method whereas the protein content was determined by the Lowry protein assay- FC method and the calcium content was volumetrically analysed with potassium permanganate titration. This study represents the first comparative analysis of CPP with different milk samples and reveals a higher level of casein, protein and calcium content of CPP than previously demonstrated. The characterisation of the CPP provided detailed insights of its nature and potential uses, these bioactive peptides may further function as health care products providing therapeutic value for either treatment of infection or prevention of a disease.

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INTRODUCTION

Milk is a unique food providing a variety of essential nutrients necessary to properly fuel the body and is primary source of complete nutritional requirement. There are many major proteins which are uniquely present in milk.These bioactive peptides may function as health care products, providing therapeutic value for either treatment of infection or prevention of disease. A bioactive peptide produced during casein digestion, CPP acts by forming a complex with soluble calcium. CPP has always been a widely studied peptide group in dentistry. (Mazzouaki, 2003).CPP has been researched in the areas of sports medicine, anti-hypertensive medicine, remineralization, immuno enhancement and immune modulation (David, 2002; Tobita, 2006).

Virtual problem associated with any food based item is that it has less shelf life, if CPP is isolated from the fermented milk, then it reduces the dissipation rate, increases shelf life and the risk involved with the microorganisms decreases considerably. Various parameters are important to be studied and standardized before commencing the work like titrable acidity and pH. Even a slight alteration in the pH can drastically affect the properties of the fermented milk resulting in the physical and chemical changes. Acidification is also an essential parameter of the fermentation process. In the case of titrable acidity, it is bound to have differences within the samples fermented with the multiple cultures of *Lactobacillus* (Kantha and Balaji, 2010).

The proteolytic system of lactic acid bacteria is essential for their growth in milk and contributes significantly to flavor development in fermented milk products where these microorganisms are used as starter cultures. The proteolytic system is composed of proteinases which initially cleave the milk protein to peptides, peptidases which cleave the peptides thus formed into smaller peptides and amino acids and transport systems which are involved in the cellular uptake of small peptides and amino acids. A wide spectrum of peptidases has been identified in lactic acid bacteria (Hans and Wilhelm, 1999**).**

CPP has a proven role as an anti-hypertensive enhancing (David, 2002).The caseinophosphopeptides (CPP) are derived from the milk protein casein by tryptic digestion yields phosphopeptides from their polar N-terminal regions. In alkaline conditions this calcium phosphate is present as an alkaline amorphous phase complexed by the CPP, referred to as caseinophosphopeptides-amorphous calcium phosphate (CPP-ACP). The CPP-ACP complexes readily incorporate fluoride ions forming caseinophosphopeptides (Cross KJ *et al.*, 2007).

The CPP have a remarkable ability to stabilize calcium phosphate in solution and substantially increase the level of calcium phosphate in dental plaque. Through their multiple phosphoseryl residues, the CPP bind to form clusters of amorphous calcium phosphate (ACP). The proposed mechanism for the CPP-ACP is that they localize ACP in dental plaque, which buffers the free calcium and phosphate ion activities, thereby helping to maintain a state of

supersaturation with respect to tooth enamel depressing demineralization and enhancing remineralization. CPP-ACP is an anti-cariogenic agent that is suitable to be added to foods and also the dentifrices or oral care products by localizing calcium and phosphate ions at the tooth surface (Reynolds EC, 1998).

It is concluded that the CPP are a safe and novel carrier for calcium, phosphate and hydroxide (fluoride) ions to promote enamel remineralization with application in oral care products, dental professional products and foodstuffs.

Casein peptides are also used for high blood pressure, high cholesterol, anxiety, fatigue, epilepsy, intestinal disorders, cancer prevention and stress reduction. Caseinophosphopeptides (CPP) also has been proposed as potential dietary antioxidants on designing new functional products (Jose Moises *et al.,* 2008).

There are many factors affecting Ca availability, such as the various co-present dietary compounds in the intestinal lumen. An important factor determining the contribution of the intestine to overall calcium absorption is the relatively long transit time of calcium. The higher absorption of calcium occurred when inorganic phosphate was added to the Ca-CPP preparation. CPP exhibit a potent ability to form soluble complexes with Ca^{2+} and other trace elements, preventing the formation of Ca-phosphate precipitate in the intestine (Gabriella Pinto *et al.*, 2012).

The heating process affects the bioavailability of CPP for example; milk sterilization can induce dephosphorylation of phosphoseryl residues and dehydroalanine residue formation. Some commercial products have been developed containing moderately hydrolysed milk proteins as the sole protein source. CPP generally had an improved solubility and transparency even under acid conditions and could be used as ingredient for beverages such as sport drinks, soft drinks, health drinks, fermented products, vitamin concentrates, fruit or fruit fractions. In addition to fluids, preparation of CPP could be used as additive for healthy foods or for dietetic or pharmaceutical compositions, as they are capable of increasing the *in vivo* absorption of calcium, total proteins and casein (Gabriella Pinto *et al.,* 2012).

Total proteins in CPP were estimated by Lowry's method for comparison with the three milk samples.The Lowry method is sensitive to low concentrations of protein.The absorbance was measured at 660 nm and the standard graph was plotted.

Casein is not heat sensitive; only temperatures up to or above 120ºC causes the casein to gradually become insoluble, whereas it is sensitive to pH and will precipitate at its isoelectric pH (Walstra *et al.,* 1999). Casein was determined by Walker method which is based on Sorenson formic method.

MATERIALS AND METHODS

Milk Fermentation

1500ml of three different milk samples from cow, goat and buffalo were taken and divided into three equal proportions containing 500ml for each. To this 5% of the cultures of *L. bulgaricus, L.casei* and *S.thermophilus* which are designated as Culture A, Culture B and Culture C were added to all the three portions in triplicate for statistical purpose.The fermentation was allowed to take place overnight.

Initial Standardisation of pH and Titrable Acidity

The two parameters pH and titrable acidity are vital for the effective fermentation of the milk and have drastic impact on production of CPP. The pH of the fermented milk samples was recorded in a time interval of 1 hour and the Arithmetic mean of the values were taken. The standard deviation was also calculated based on the mean value. The titrable acidity of all the fermented milk samples was determined titrimetrically using 0.1M NaOH with phenolphthalein as an indicator and it represented the used up milli liters of a 0.1M NaOH for neutralization of the acid in 10ml of the product.

Isolation of Caseinophosphopeptides (CPP)

The milk samples which were fermented for 24 hours were taken and the pH was adjusted to 7.0 using 0.5M NaOH. 5% casein suspension was prepared by mixing casein in a magnetic stirrer. After the pH was adjusted to 7.0 enzyme trypsin at Enzyme: Substrate ratio of 1:100 was added. Then hydrolysis was carried out by mixing the suspension in a water bath using magnetic stirrer at 37^0C for 30 minutes. The pH of the solution was kept constant at pH 7.0 by addition of 0.1M NaOH solution.

After complete hydrolysis, the mixture was removed from the water bath. The pH of this casein hydrolysate was readjusted to 4.6 using 2M HCl. Centrifugation of this mixture was done at 3000rpm for 10 minutes to remove the non-phosphorylated peptides. The supernatant obtained was removed and the pH was adjusted to 7.0 using 2M NaOH. Calcium chloride at1% level was added to the supernatant and allowed to stand for 1 hour at room temperature. 50% (V/V) ethanol was added to the above and the precipitate was collected by centrifugation at 6000rpm for 10 minutes. The CPPs thus obtained were air dried and stored.

The above given procedure was done to isolate the CPP from all the three different milk samples (Kantha D and R Balaji 2010).

Determination of the total protein

0.2 ml of BSA working standard was taken in 5 test tubes and made up to 1ml using distilled water. The test tube with 1 ml distilled water served as blank. 4.5 ml of alkaline copper reagent was added and incubated for 10 minutes.After incubation 0.5 ml of Folin Ciocalteau (FC) reagent was added and incubated for 30 minutes. The absorbance was measured at 660 nm and the standard graph was plotted. 0.01ml of the test solution was taken in a test tube and made up to 1ml using distilled water and the above steps were repeated for each sample (Dunn 1992).

Determination of the total casein content

To 10ml of the CPP solution, 0.5 ml of 2% alcohol dilution of phenolphthalein was added and titrated against 0.1 M NaOH (sodium hydroxide) up to the appearance of pale pink color. This was followed by the addition of 2 ml of 20% formalin and titrated once more against 0.1 M NaOH up to the appearance of pale pink colour, as at the first time (F. H. McDowall and A. K. R. McDowell 1936).

The casein content is calculated according to equation:

X = a x 1.47

Where: a – amount of 0.1 M NaOH used for titration after formalin addition

1.47 – counting factor for casein

then transferred into a 100ml standard flask and made upto the mark with distilled water. 10ml of the filtrate was pipetted out into a conical flask and 4% ammonium oxalate was added drop wise till a cloudy precipitate was formed and kept overnight.

Table 1 Comparison of pH of the cow, goat and buffalo milk sample with *Lactic Acid Bacteria* (LAB).

	pH (MEAN ± S.D)								
	Cow sample			Goat sample			Buffalo sample		
TIME (hours)	*L. bulgaricus*	*L. casei*	*S. thermophilus*	*L. bulgaricus*	*L. casei*	*S. thermophilus*	*L. bulgaricus*	*L. casei*	*S. thermophilus*
1	5.0± 0.12	6.0±0.18	6.5±0.18	6.5±0.18	6.0±0.15	6.5±0.18	7.0±0.20	6.0±0.15	7.0±0.18
2	5.0± 0.14	6.0±0.18	7.0±0.19	7.0±0.20	6.5±0.18	6.5±0.19	7.0±0.21	6.0±0.14	7.0±0.20
3	4.0±0.16	4.0±0.16	7.0±0.19	7.0±0.21	6.8±0.20	6.5±0.18	6.0±0.15	6.0±0.15	6.0±0.12
4	3.0±0.14	3.0±0.14	7.0±0.19	7.0±0.20	6.8±0.19	6.8±0.20	6.5±0.17	6.0±0.14	6.5±0.17

Table 2 Comparison of titrable acidity of the cow, goat and buffalo milk sample with *Lactic Acid Bacteria* (LAB).

	PERCENTAGE OF ACIDITY (MEAN±S.D)		
	Cow milk	Goat milk	Buffalo milk
L. bulgaricus	0.09±0.12	3.06±0.22	2.25±0.19
L. casei	0.13±0.12	2.16±0.20	2.52±0.18
S. thermophilus	0.15±0.14	2.43±0.21	2.25±0.16

Table 3 Comparison of the total protein content of CPP from cow, goat and buffalo milk sample fermented with different strains of LAB.

	AMOUNT OF TOTAL PROTEIN (mg/ml)		
	Cow milk	Goat milk	Buffalo milk
L. bulgaricus	41.6±0.20	23.6±0.17	8.0±0.14
L. casei	30.4±0.19	16.4±0.16	2.8±0.13
S. thermophilus	28.4±0.17	25.6±0.18	7.2±0.14

Table 4 Comparison of the casein content of CPP from cow, goat and buffalo milk sample fermented with different strains of LAB.

	PERCENTAGE OF CASEIN (MEAN±S.D)		
	Cow milk	Goat milk	Buffalo milk
L. bulgaricus	1.47±0.12	6.61±0.19	2.94±0.16
L. casei	1.47±0.12	2.94±0.20	2.94±0.16
S. thermophilus	2.80±0.18	1.47±0.16	7.35±0.20

Table 5 Comparison of Calcium content of CPP from cow, goat and buffalo milk sample fermented with different strains of LAB.

	AMOUNT OF CALCIUM (grams/100 ml)		
	Cow milk	Goat milk	Buffalo milk
L. bulgaricus	1.12±0.14	0.48±0.13	0.32±0.14
L. casei	0.72±0.12	0.60±0.14	0.28±0.13
S. thermophilus	0.32±0.12	0.20±0.12	0.20±0.12

Determination of the calcium content of the CPP

20ml of CPP solution was taken in a beaker and equal volume of water was added and mixed thoroughly with 2ml of 10% acetic acid. It was stirred continuously with the precipitate that was formed.

At this point the reaction mixture was acidic to litmus. The solution was boiled for 2-3 minutes. It was then filtered and the precipitate formed was washed with hot water to dissolve calcium into the solution from the precipitate. The filtrate was

The solution was filtered with Whatmann filter paper and the precipitate along with the filter paper was dissolved in 10ml of sulphuric acid and mashed.

This was heated till the first bubble appeared. The liberated oxalic acid was then titrated against permanganate. The duplicate was also prepared in the same manner and titrated. From the values obtained the amount of calcium present in the given sample was calculated (Eugene Y.B 1955).

RESULTS AND DISCUSSION

Determination of pH and titrable acidity

The pH was estimated for all the milk samples post fermentation at the interval of one hour. The values were compared with the samples fermented with different strains of LAB. *S. thermophilus* showed high pH 6.8 to 7.0 in all the samples while *L.bulgaricus* showed highest variations from pH 3.0 to 7.0. *L.casei* showed least variations and consistent pH from 6.0 to 6.5 in all samples (Figure 1).

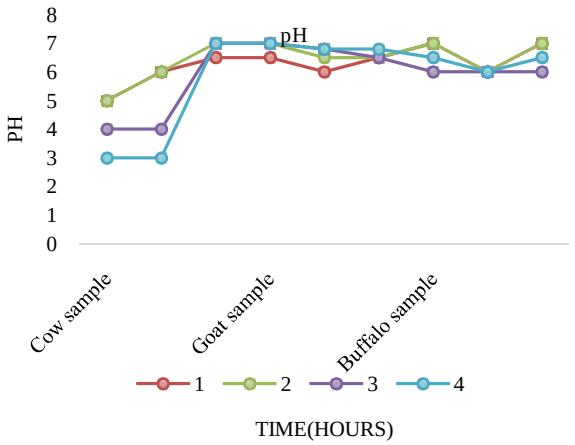

Figure 1 Comparison of pH of the cow, goat and buffalo milk sample fermented by *Lactic Acid Bacteria* (LAB)

S. thermophilus showed the highest amountof lactic acid production in all the milk samples when compared to *L.casei* which showed a slightly lower production of the lactic acid.

L.bulgaricus showed the least production of lactic acid in the samples. The milk sample which showed the highest acid production post fermentation was buffalo milk.The least production of acid was seen in the cow milk (Figure 2).

The maximum amount of CPP was obtained from the Cow milk sample.

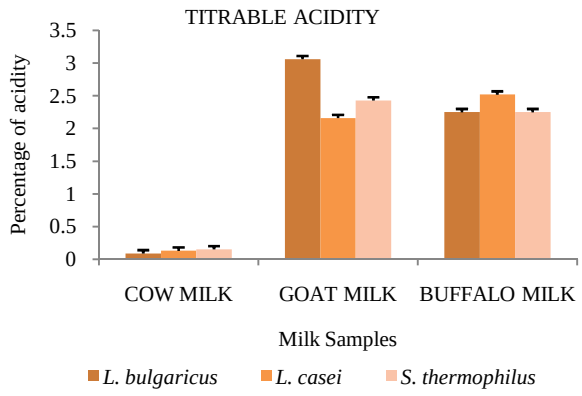

Figure 2 Comparison of titrable acidity of different milk samples Post fermentation.

Determination of casein content of CPP

The CPP obtained from milk samples fermented with *S.thermophilus*showed the highest casein content whereas *L. bulgaricus* showed slightly lower casein content. The milk samples fermented with *L. casei* showed the least casein content. The CPP obtained from buffalo milk showed the highest casein content whereas the least was shown by the CPP obtained from cow milk (Figure 3).

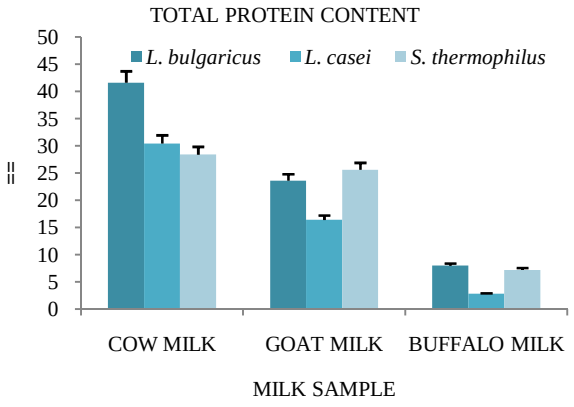

Figure 3 Comparison of total protein content of CPP

Determination of Total Protein of CPP

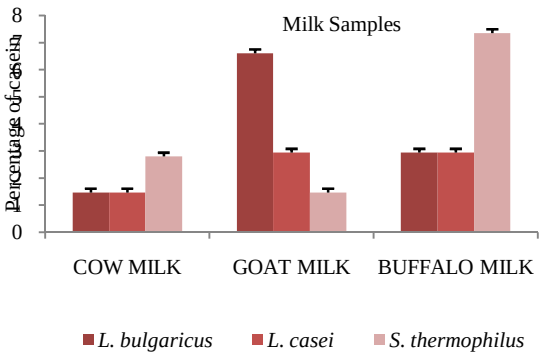

Figure 4 Comparison of percentage of casein of CPP.

The Total protein content (mg/ml) was found to be the highest in the CPP obtained from the milk fermented with *L. bulgaricus* whereas the least protein content was shown by the CPP obtained from milk sample fermented with *L. casei*. The highest total protein content was shown by the CPP obtained from the fermented cow milk sample while the least content was shown by the CPP from fermented buffalo milk (Figure 4).

Analysis of calcium content of CPP

The calcium content in the CPP obtained from the milk sample fermented with *L.bulgaricus* showed the highest calcium content whereas the least calcium content was shown by the CPP from milk sample fermented with *S.thermophilus*.The highest calcium content (grams/100ml) was shown in fermented cow milk sample.The least content was shown in fermented buffalo milk sample (Figure 5).

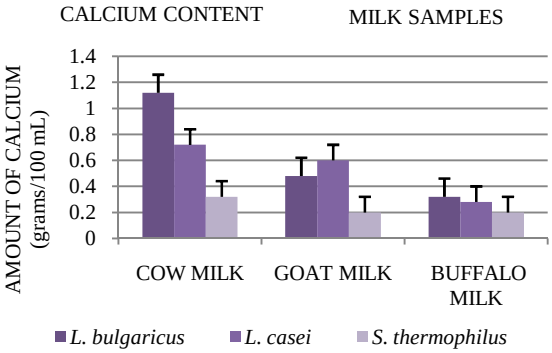

Figure 5 Comparison of calcium content of CPP

Caseinophosphopeptides (CPP) are one of the many nutraceuticals isolated from fermented milk which are majorly used for treatment and prevention of dental caries.CPP has a proven role as an anti-hypertensive enhancing agent and have got immunomodulatory activity.CPP have been commonly incorporated in commercial products like toothpaste, chewing gum and also used in other confectioneries for oral health care (Kantha and Balaji, 2010).

Also CPP can be administered as a milk based enriched supplement of calcium and proteins to lactose intolerant individuals. CPP incorporated in functional foods promotes beneficial effects on human health such as recalcification of bones, protecting the tooth enamel from decay and many other possible health benefits. Milk and dairy products provide plenty of Ca2+, which can form soluble complexes with those phosphopeptides, (Sato, Naguchi, & Naito, 1986; Berrocal *et al*., 1989)and enhancing intestinal absorption of Ca and retentionthereof in the body (Mykkanen& Wasserman, 1980; Sato *et al*., 1986).CPP used as an ingredient in food are also targeted for prevention of osteoporosis that is by increased absorption of calcium. CPP are used insports medicine and their roles in that field also can bewidened. The fermented milk peptides play a natural rolein many biochemical and immunological mechanismsinside the human body and they can be formulated in toeffective oral medicine for all age groups (Kantha and Balaji, 2010).

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