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In Silico and *In vitro* Analysis of Novel Angiotensin I-Converting Enzyme (ACE) inhibitory Bioactive Peptides Derived from Fermented Camel Milk (*Camelus dromedarius*)

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Abstract In this study, *Lactobacillus bulgaricus* NCDC (09) and *Lactobacillus fermentum* TDS030603 (LBF) were evaluated for their ACE-inhibitory activity and peptides production under optimized conditions from fermented camel milk (*Camelus dromedarius*). Lactic cultures were evaluated for their pepX activity, proteolytic activity and ACE-inhibitory activity. 09 culture exhibited higher PepX and ACE-inhibitory activity than LBF. 2% rate of inoculation and 12 h of incubation were optimized on the basis of pepX and proteolytic activity. Purified peptides from fermented camel milk were characterized by amino acids profiling through the search in BlastP, Protein information resource (PIR) databases. ACE-inhibitory activity of different peptides from fermented camel milk were also confirmed by the database of antihypertensive peptides (AHTPDB). Fermented camel milk produced by Lactobacillus cultures could be a novel source of ACE-inhibitory peptides.

Keywords Fermented camel milk · ACE-inhibitory activity · Peptide production and identification

Introduction

Milk proteins are known for many biological activities that play significant role in improving human health and nutrition (Park 2009). Bioactive peptides can be produced from milk proteins by digestive enzymes (proteases), microbial or plant enzymes or by fermenting with proteolytic dairy starter cultures (Hayes et al. 2007). Bioactive peptides are well-known with health benefits like cholesterol-lowering (Hartmann and Meisel 2007), antimicrobial (López-Expósito and Recio 2006), immunomodulatory, opioid, antioxidative, antihypertensive (Jäkälä and Vapaatalo 2010) and mineral-binding (Vegarud et al. 2000). The main bioactive peptides studied across the world are those with antihypertensive effect (Korhonen and Pihlanto 2006), which are known for their angiotensin-converting enzyme (ACE) inhibiting function. ACE-inhibitions plays key role in blood pressure regulation (De Leo et al. 2009). It has been reported that, milk derived ACE-inhibitory peptides exhibit the inhibition of conversion of angiotensin I to angiotensin II; thus these peptides have a blood pressure lowering effect (Vermeirssen et al. 2004). ACE-inhibitory peptides have been isolated from variety of fermented dairy products including yoghurt (Donkor 2007), cheese (Hartmann and Meisel 2007) and fermented bovine milk (Qian et al. 2011). Use of proteolytic lactic acid bacteria for the milk fermentation is one of the economical and practical methods for the production of fermented dairy products enriched with bioactive peptides (Hayes et al. 2007). The proteolytic system of LAB can contribute to the liberation of health enhancing bioactive peptides from milk. The latter may improve absorption in the intestinal tract, stimulate the immune system, exert antihypertensive or antithrombotic effects and antioxidative activity or function as carriers for minerals especially calcium (Padghan et al. 2016). As per the previous reports, bovine milk fermented with different proteolytic lactic acid bacterial strains such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (Papadimitriou et al. 2007), *Lactobacillus helveticus* JCM1004, *Lactobacillus helveticus* MTCC5463 (Pan et al. 2005; Hati et al. 2016), *Lactobacillus acidophilus* DPC6026 and *Lactococcus*

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lactis YT2027 contain health-beneficial bioactive peptides. Fermented milk products are produced from different species like bovine, goat, sheep, buffalo, etc (Chandan 2004). Camel milk is well suitable for fermented milk production. It contains well-balanced nutrients and biological components. Camel milk is high in vitamin C and niacin, as well as richer in Cu and Fe than bovine milk (El-Agamy 2006). Moreover, camel milk lacks beta-lactoglobulin and contains alpha-lactalbumin, a similar situation to that in human milk. Fermented camel milk is now available in different countries and traditionally produced by the communities. In Ethiopia, dromedary camel milk was used as a potential treatment for a number of diseases like Malaria, Jaundice, to clear the stomach, flatulence, to detoxify snake venom, constipation and post pregnancy care of women (Seifu 2007; Mehari et al. 2007). Also used in treating other health problems such as dropsy, tuberculosis, asthma and leishmaniasis or kala-azar in different parts of the world including India, Russia and Sudan (Abdelgadir et al. 1998). Fermented camel milk (FCM) is also considered as probiotic with its unique antibodies and medicinal properties. FCM may provide beneficial effects like anti-inflammation and anti-obesity (Badkook 2013). Fermented camel milk is also a source of different bioactive peptides. Bioactive peptides like ACE-inhibitory peptides and antioxidative peptides were also derived from fermented camel milk (Moslehishad et al. 2013). Furthermore, some reports are available on peptides with a wide range of functionalities in fermented bovine milk with proteolytic strains of LAB (FitzGerald and Murray 2006; Hayes et al. 2007; Papadimitriou et al. 2007), but there is a lack of information in the literature about biological activity of camel milk-derived peptides by lactic acid fermentation. Therefore, it would be interesting to investigate the functionalities of bioactive peptides produced during the fermentation of camel milk. In this study, *L. bulgaricus* NCDC (09) and *L. fermentum* TDS030603 (LBF) were considered because of their high proteolytic activity and ACE-inhibitory activity in camel milk medium. The objective of this study was to purify and characterize the ACE-inhibitory peptides derived from fermented camel milk (*Camelus dromedarius*).

Materials and Methods

Collection of Lactobacillus Cultures and their Maintenance

The LAB cultures used in the present study i.e. *L. bulgaricus* NCDC (09) and *L. fermentum* TDS030603 (LBF) were obtained from the Culture Collection of Dairy Microbiology Department, SMC College of Dairy Science, Anand, Gujarat, India. The lactic cultures were propagated in sterilized reconstituted skim milk (10% TS) and stored at 5 ± 2 °C. The transfer was given every week during the course of the study. Purity and activity was tested every time before sub-culturing.

Camel Milk Procurement

Fresh camel milk (*Camelus dromedarius*) sample was procured from a farm at Ankalav village of Anand District, Gujarat, India.

Assessment of X-prolyl-dipeptidyl Aminopeptidase Activity (PepX)

Pure lactic cultures were activated in MRS broth at the rate of 2% of inoculation and incubated for 24 h at 37 °C. Freshly grown cultures were inoculated at the rate of 2% in 100 mL MRS broth and incubated for 0, 3, 6, 9 and 12 h. Pellets were collected after centrifugation at 3500 rpm for 15 min at 4 °C. (Hermle centrifuge, Germany). Then, pellets were washed with 25 mL of sodium phosphate buffer and finally pellets were dissolved in 10 mL of sodium phosphate buffer. Then cell pellets were sonicated by probe sonicator (Labman, India). After sonication, the supernatants were collected by centrifugation at 13,000 rpm for 13 min at 4 °C. Then, crude supernatants collected were used as samples for the enzymatic assay.

Enzymatic Reaction of pepX Activity

X-prolyl-dipeptidyl aminopeptidase (pepX) activity of the cultures was assayed with glycyl-prolyl *p*-nitroanilide (Sigma, USA) (Gly-Pro-pNA) as the substrate with some modifications (Donkor et al. 2007). The incubation mixture contained 50 µL of 6.4 mmol/L of substrate glycyl-prolyl *p*-nitroanilide, 2.85 mL of 50 mmol L^{-1} of Tris–HCl buffer (pH 7.0) and 100 µL of sample (supernatant). The mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 500 µL of 30% acetic acid. The extent of hydrolysis was measured with the spectrophotometer (Systronics Spectrophotometer 2202, India) at 410 nm. The activity of each sample was evaluated in triplicate.

Preparation of Fermented Camel Milk

Fresh camel milk was collected and then filtered through muslin cloth and heated at 90 °C for 10 min and then stored at 5 ± 1 °C. 2% of previously grown active cultures from reconstituted milk were inoculated in camel milk and incubated for 24 h at 37 °C for activation in camel milk medium.

Preparation of the Supernatant (Water Soluble Extract) from Fermented Camel Milk

Fermented camel milk samples were centrifuged at 13,000 rpm for 20 min at 4 °C (Hermle centrifuge, Germany). The supernatant (Water soluble extract) was collected and it was filtered through 0.45 µm membrane filter (Millex® - HV, MERK, Ireland). This supernatant was further assayed for ACE-Inhibitory activity.

Determination of ACE-inhibitory Activity

The ACE-inhibitory activity percentage (ACEi %) was determined according to the method described by Hati et al. (2015) with some modifications as described below. 50 µL of 5 mM HHL (Sigma, USA) (10.74 mg HHL in 5 mL sodium borate buffer, pH 8.3) solution was mixed with 500 µL deionized water and 200 µL of sample (Water soluble extract). The residue containing hippuric acid was dissolved in 2 mL of deionized water and it was filtered through 0.45 µm membrane filter (Millex® - HV, MERK Ireland). The absorbance of the solution was measured spectrophotometrically (Systronics Spectrophotometer 2202, India) at 228 nm. The activity of each sample was tested in triplicate. The formula used for the determination of ACE-inhibitory peptides is such as; $ACEi\% = \{(Absorbance\ of\ HA_{control} - Absorbance\ of\ HA_{sample}/Absorbance\ of\ HA_{control}) \times 100\}$. Where, HA control was the absorbance of concentration of hippuric acid produced by the ACE in buffer without lactic cultures. HA sample was the absorbance of the concentration of hippuric acid produced by the ACE in the presence of lactic cultures. In the case of fractionated 3, 5 and 10 kDa, HA control was the absorbance of concentration of hippuric acid produced by the ACE in camel milk without lactic cultures. HA sample was the absorbance of the concentration of hippuric acid produced by the ACE in the presence of lactic cultures in camel milk medium.

Determination of Proteolytic Activity of Lactic Cultures in Camel Milk Medium

Growth conditions for the production peptides of the selected lactic acid bacteria was optimized by measuring the proteolytic activity through o-phthaldialdehyde (OPA) method (Hati et al. 2016).

Sample Preparation for the Proteolytic Activity

Both the lactic cultures were activated by growing in heat treated camel milk. Different rates of inoculation like 1.0, 1.5 and 2% were used with different incubation periods to optimize the growth conditions for the production of peptides. The culture tubes were incubated at 37 °C for different intervals i.e. 0, 3, 6, 9 and 12 h, the tubes were taken out for the evaluation of peptide content (Proteolytic activity) after each interval. The degree of proteolysis during fermentation of milk was determined by measuring the release of free NH_3 groups following the o-phthaldialdehyde (OPA) method (Hati et al. 2016).

Enzymatic Reaction of Proteolytic Activity

An aliquot of 3 mL from each fermented camel milk sample was mixed with 5 mL of 0.75% tri-chloroacetic acid (TCA) and vortex for 1 min, and then the mixture was filtered using Whatman™ no. 42 filter papers (UK). The filtrate (200 µL) was added to 3 mL of OPA reagent and after the incubation at room temperature (20 °C) for 2 min, absorbance of the solution was measured by a spectrophotometer (Systronics PC based double beam Spectrophotometer 2202, India) at 340 nm. The proteolytic activity of these lactic cultures was expressed as the absorbance of free amino groups measured at 340 nm. A relative degree of proteolysis was determined as the difference between proteolytic activities in fermented camel milk to that of unfermented camel milk. All the analyses were carried out in triplicate.

Peptides Purification by RP-HPLC

Relative proteolytic activity was assessed by peptides mapping of the fermented camel milks, performed using a RP-HPLC (Shimadzu LC-20, Japan), for the separation of different peaks. A binary gradient RP-HPLC system was used, fitted with C_{18} column (Se Quant® ZIC®-cHILIC) white pore analytic column (3 µ, 250×4.6 mm). Sample was applied using microinjector (HAMILTON Bonaduz AG, Switzerland) with 20 µL loop. Eluent-A was 1% (v/v) of TFA in deionized water and Eluent-B was 1% (v/v) of TFA in mixture of 80:20 of acetonitrile and deionized water. Separation was conducted at room temperature at flow rate 0.25 mL/min with eluent-A for 0–10 min. and linear gradient, from 0 to 80% of eluent-B, for 0–10 min. The column finally eluted with 100% eluent-B for 11–20 min. Peaks were a function of absorbance observed by an ultra violet/visible wavelength detector (Shimadzu, SPD-20A) operating at 214 nm. Total peak area was obtained by the integration of all the peaks observed. All the analysis were carried out in triplicate. The change of peptide profile was expressed as relative proteolytic activity, R*pa* %, calculated from the given equation as; $Rpa\% = \{(TPE_b - TPE_e/TPE_e) \times 100\}$. Where TPE_b = Total peak area at the beginning (unfermented camel milk sample), TPE_e = Total peak area at the end of hydrolysis (fermented camel milk sample).

Amino Acid Characterization of ACE-Inhibitory Peptides Through RP-LC/MS

Fractionations of 3 kDa permeate and 10 kDa permeate samples was carried out through RP-HPLC. Each of the sample was injected to collect different fractions eluted at different time intervals (retention times) and these fractions were used for the characterization of amino acid sequence of the peptides through RP-LC/MS. Ekspert ultra LC 100 (Eksigent, USA) was used in conjunction with ABSCIEX QTRAP 4500 Ion Trap Mass Spectrometer via Electron Spray Ionization (ESI) interface. In built MASCOT script was used for the characterization of the peptides following the protocols given by Jakubczyk and Baraniak (2014); Tagliazucchi et al. (2015) with some modifications (viz., search parameter such as peptide mass tolerance set was 1.2 and MS/MS tolerance was 0.6). RP-LC/MS was used for identification of peptides through Enhanced Mass Spectra (EMS) following three Enhanced Product Ion (EPI) scan using Information Dependent Analysis (IDA) survey scan.

Chromatography

The sequence analysis of peptides in the fractions of 3 and 10 kDa permeates were carried out using liquid chromatography (LC) connected to a mass spectrometer (MS). For liquid chromatography, Ekspert ultra LC 100 from Eksigent, USA was used in conjunction with ABSCIEX QTRAP 4500 ion trap mass spectrometer via Electron Spray Ionization (ESI) interface. The LC-100 was equipped with degasser, quaternary pump, column oven and auto-sampler. Gradient elution was optimized for getting good resolution and noise control during acquisition. For Liquid Chromatography ACQUITY UPLC-BEH C_{18} (2.1×50 mm, 1.7 µm) column (WATERS Company make, UK) was used. The column temperature and sample temperature were maintained at 65 and 4 °C respectively. The mobile phase was composed of solvent A (water) and solvent B (Acetonitrile) with 0.1% formic acid each. The gradient column elution was as follows: (i) 0 min, 95% A and 5% B; (ii) 0–2 min, 90% A and 10% B; (iii) 2–15 min, 50% A and 50% B; (iv) 15–22 min, 50% A and 50% B; (v) 22–24 min, 10% A and 90% B; (vi) 24–25 min, 10% A and 90% B; (vii) 25–35 min, 10% A and 90% B and (viii) 35–60 min, 95% A and 5% B. The flow rate of the mobile phase was set to 0.4 mL/min with an injection volume fixed at 5 µL.

Peptide Identification (Mass Spectrometry MS/MS)

Identification of peptides sequences from fractions exhibiting remarkable ACE-inhibitory activity was carried out by RP-LC/MS. In this study an Information Dependent Analysis (IDA) workflow was optimized for protein identification consisting EMS scan followed by 3 EPI scan. IDA was set by providing Rolling Collision Energy excluding former target ion for 20 s. Optimized IDA methods for identification of peptides was given in Table 1.

Data Processing

Acquired raw data were processed by Analyst software followed by Mascot Search (Matrix Science, London, UK, onsite license) against Swiss-Prot database. Search parameters for peptide and product ions mass tolerance were 1.2 and 0.6 Da, respectively. Enzyme specificity was no cleavage for HPLC purified undigested samples with missed cleavage sites allowed were 0, and fixed modification of cysteine was by carbamidomethylation and variable modification was set to carboxymethylation and methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to <5% false positive rate, calculated by Mascot procedure, and with the Mascot significant score were considered to be positively identified.

In Silico Analysis

Peptide sequences derived from MASCOT with significant score were matched in the databases with the Taxonomy ID-9838 for one humped camel milk protein (*Camelus dromedarius*) database search against (I) NCBI (blastp tool) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) (II) Protein Information Resource (PIR) to confirm the camel milk

Table 1 Optimized parameters for EMS to EPI survey scan with IDA criteria for identification of peptides

EMS	Start mass (Da)	Stop mass (Da)	LIT
	400	2000	20 ms
EPI	100	2000	50 ms
Gas-1 (N_2)	50	Temperature (TEM)	300
Gas-2 (N_2)	40	Collision induced dissociation (CAD)	High
Potential of cone (DP)	50 V	Curtain gas (CUR)	30
Collision energy (CE)	10 V	Ion spray voltage (IPV)	5500
Entrance potential (EP)	10	Collision energy sprade (CES)	0

protein sequences (3–30 amino acids) (http://research.bioinformatics.udel.edu/peptidematch/index.jsp) (Barker et al. 2001). Also, the peptide sequences were matched with the Database of Antihypertensive peptides (AHTPDB) for confirming the ACE-inhibitory activity (http://crdd.osdd.net/raghava/ahtpdb/pep.php) (Kumar et al. 2015).

Statistical Analysis

All the parameters under the study were analyzed by statistical methods. All determinations were performed at least in triplicate, and the results were expressed as means ± standard deviations (SD). Data obtained were analyzed by statistical designs and softwares. Significant differences between treatments were tested by analysis of variance (ANOVA) with a level of significance of $p < 0.05$.

Results and Discussions

Assessment of X-prolyl-dipeptidyl Aminopeptidase Activity (PepX)

Both the lactic cultures were activated individually in sterilized reconstituted skim milk at 2% rate of inoculation and incubated for 24 h at 37 °C. The PepX activity was evaluated for growing all the active lactic cultures in MRS broth, by inoculating at the rate of 2% (v/v) and incubating for 0, 3, 6, 9 and 12 h at 37 °C. As the PepX is an intracellular enzyme, we had taken intracellular extracts which may be have higher PepX activity as compared to extracellular extract for the same cultures in a same medium. The PepX activity was analyzed spectrophotometrically as absorbance at 410 nm (optical density). X-prolyl-dipeptidyl aminopeptidase (PepX) activity of dairy lactic cultures is an important characteristic due to high proline content in milk protein. The capability of PepX is to cleave Xaa-Pro dipeptides from the (X-Pro ↓ Y…) N terminus of peptides (Pan et al. 2005). PepX releases Xaa-Pro dipeptides from peptides ranging from three to seven amino acid residues; the exhibition of PepX activity from lactic acid bacteria indicates its ability to produce bioactive peptides (Pan et al. 2005). Different studies had been reported about the PepX activity of LAB in milk and milk products. Extracellular and Intracellular extracts were used to determine the PepX activity. PepX activity of both the culture was presented in the Table 2. PepX activity was differing significantly ($p < 0.05$) with incubation periods. Also, there was a significant difference ($p < 0.05$) observed within the cultures. Also it was found that, the PepX activity of both the lactic cultures was increased significantly with the time of incubation.

Pastar et al. (2003) and Savijoki et al. (2006) also reported that, *Lb. rhamnosus* possesses a proteolytic system that includes a proline-specific peptidase like proline-specific aminopeptidase (PepR) and X-prolyl-dipeptidyl aminopeptidase (PepX) that subsequently may result in accumulation of bioactive ACE-inhibitors in fermented milk. Pan et al. (2005) similarly identified X-prolyl-dipeptidyl aminopeptidase activity from IE cell-free extract of *L. helveticus* JCM1004 in the hydrolysis of skimmed milk proteins and expressed as 23.6 ± 1.6 units per gram. Degraeve and Martial (2003) also reported the PepX acticity of *Lactobacillus helveticus* ITG LH1 and expressed as 35 ± 7 Ug.protein^{-1}, a strain used for Swiss-type cheese and also purified X-prolyl dipeptidyl aminopeptidase. It was also reported that, the proline specific aminopeptidases such as prolyl iminopeptidases (E.C. 3.4.11.5) and X-prolyl dipeptidyl aminopeptidases (E.C. 3.4.14.5) were found in *Streptococcus thermophilus, Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* which were used as a starter bacteria in Swiss-type cheese production (Degraeve and Martial 2003). Kunji et al. (1996) also reported the presence of pepX in all species of LAB.

Determination of ACE-inhibitory Activity

In this study, the ACE-inhibitory activity was assessed by fermenting heat treated camel milk (90 °C for 10 min.) with two pure lactic cultures at the rate of 2% inoculation, incubated at 37 °C for different time intervals (0, 12, 24 and 48 h). Different intervals 0, 3, 6 and 9 h of incubation was also tested to check %ACE-inhibitory activity but it showed very less difference in % ACE-inhibitory activity, so, finally the periods of 0, 12, 24 and 48 h of incubation was tested. ACE-inhibitory activity was differing significantly ($p < 0.05$) with incubation periods. Also, there was a significant difference ($p < 0.05$) observed within the

Table 2 PepX activity of lactic cultures incubated at 37 °C up to 12 h

Lactic cultures	Period (time in hours)				
	0	3	6	9	12
	PepX activity (O.D at 410 nm)				
09	0.209 ± 0.003	0.334 ± 0.006	0.514 ± 0.006	0.809 ± 0.029	0.893 ± 0.009
LBF	0.267 ± 0.007	0.390 ± 0.001	0.549 ± 0.024	0.718 ± 0.085	0.873 ± 0.048

cultures. ACE-inhibitory inhibitory activity was increased from 0 to 48 h in both the lactic cultures. This might be suggested that, the ACE-inhibitory activity was increasing with the growth of lactic cultures during their growth. It was observed that, ACE-inhibitory activity was differing significantly ($p < 0.05$) with incubation periods (Table 3). Also, there was a significant difference ($p < 0.05$) observed within the cultures.

The measured ACE-inhibitory activity had been varied from 55.66 to 76.75% during the incubation period of 0–48 h (Table 3). It was also observed that, the ACE-inhibitory activity was increased from 0 to 48 h in all the lactic cultures. 09 exhibited highest ACE-inhibitory activity (76.75%) than LBF (73.93%) ACE-inhibitory activity up to 48 h of incubation period at 37 °C. This might be suggested that, the ACE-inhibitory activity was increasing with the growth of lactic cultures during their growth. Moslehishad et al. (2013) studied the ACE-inhibitory activity of camel milk and bovine milk fermented by *Lb. rhamnosus* PTCC 1637. They utilized water soluble extract as a sample, similar to our study and indicated that the IC_{50} values, especially in camel milk (2.223 ± 0.052–3.930 ± 0.118 mg mL^{-1}), were considerably lower than that reported by Chen et al. (2010) for koumiss whey (52.47 ± 2.87 mg mL^{-1}). They also suggested that, the more pronounced ACE-inhibitory activity of camel milk than bovine milk leads to the development of fermented camel milk by LAB as a novel food product containing ACE-inhibitory peptides. Hati et al. (2015) studied the ACE-inhibitory activity of *Lactobacillus rhamnosus* (NS4 and NS6), *Lactobacillus helveticus* MTCC 5463 (V3), *Lactobacillus delbruckii* (09), *Enterococcus feacalis* (ND3), *Enterococcus feacalis* (ND11), *Lactobacillus rhamnosus* (SH8) and *Lactobacillus rhamnosus* (I4) in skim milk. The results indicated that, the production of ACE-I inhibitors was not confined to single species or strain of bacteria but all the strains tested, produced peptide, which showed in-vitro ACE-I-inhibitory activity. *L. rhamnosus* (NS4) and *L. bulgaricus* (09) gave maximum ACE-I inhibitory activity 79.66 and 67.09% respectively compared to other isolates. Same isolate 09 gave exhibited highest ACE-inhibitory compared to skim milk after comparing our data with Hati et al. (2015). In our study, it may also be suggested that camel milk become a unique growth medium for the growth of lactic acid bacteria which exhibited higher ACE-inhibitory activity as compared to skim milk medium.

Table 3 ACE-inhibitory activity of lactic cultures incubated at 37 °C up to 48 h

Lactic cultures	Period (time in hours)			
	0	12	24	48
	% ACE-inhibitory activity			
09	56.34 ± 1.24	74.71 ± 1.83	74.93 ± 0.05	76.75 ± 1.14
LBF	55.66 ± 0.73	69.66 ± 5.08	71.84 ± 1.94	73.93 ± 0.74

Determination of Proteolytic Activity

Selected proteolytic lactic cultures i.e. *L. bulgaricus* NCDC (09) and *L. fermentum* TDS030603 (LBF) were used to determine the growth conditions for the production of

Table 4 Effect of proteolytic activity of 09 on inoculation rates and incubation periods at 37 °C in camel milk

Lactic culture 09	Period (Time in hours)*(P)				
	0	3	6	9	12
	Proteolytic activity (O.D. at 340 nm)				
1%	0.590 ± 0.037[a]	0.628 ± 0.007[a]	0.695 ± 0.020[a]	0.819 ± 0.016[a]	0.851 ± 0.023[b]
1.5%	0.558 ± 0.063[a]	0.658 ± 0.120[a]	0.750 ± 0.021[a]	0.842 ± 0.038[a]	0.870 ± 0.021[b]
2%	0.572 ± 0.023[a]	0.601 ± 0.003[a]	0.738 ± 0.039[a]	0.903 ± 0.108[a]	1.025 ± 0.031[a]

*Values with different superscripts differ significantly ($p < 0.05$)

Table 5 Effect of proteolytic activity of LBF on inoculation rates and incubation periods at 37 °C in camel milk

Lactic culture LBF	Period (time in hours)*(P)				
	0	3	6	9	12
	Proteolytic activity (O.D. at 340 nm)				
1%	0.542 ± 0.008[a]	0.573 ± 0.010[a]	0.600 ± 0.012[b]	0.691 ± 0.017[b]	0.745 ± 0.027[b]
1.5%	0.571 ± 0.011[a]	0.597 ± 0.005[a]	0.618 ± 0.023[b]	0.794 ± 0.028[a]	1.016 ± 0.027[a]
2%	0.598 ± 0.012[a]	0.640 ± 0.015[a]	0.819 ± 0.017[b]	0.844 ± 0.022[a]	1.023 ± 0.160[a]

*Values with different superscripts differ significantly ($p < 0.05$)

Table 6 Peptide content (%R*pa*) of lactic cultures inoculated at the rate of 2% and incubated at 37 °C for 12 h in camel milk

Lactic cultures	Peptide production (%)
LBF	41.81 ± 1.469^{b}
09	47.50 ± 1.291^{a}

Values with different superscripts differ significantly ($p < 0.05$)

peptides through the measurement of proteolytic activity. Both the cultures were inoculated at the rate of 1, 1.5 and 2% (v/v) in heat treated camel milk and incubated at 37 °C for 0, 3, 6, 9 and 12 h. Then, the sample prepared was used to optimize the growth conditions through OPA method (Hati et al. 2015). The proteolytic activity of these lactic cultures was expressed as the amount of free amino groups measured as difference in absorbance values at 340 nm, after subtraction of values for the un inoculated control camel milk.

The OPA-based spectrophotometric assay detects released α-amino groups, which result from the proteolysis of milk proteins, thus giving a direct measurement of proteolytic activity. Individually each lactic culture 09 and LBF were statistically analyzed for the optimization of growth condition for the production of peptides through the best inoculation rate (1.0, 1.5 and 2.0%) and the optimum incubation period. Peptide production of individual lactic cultures 09 and LBF in heat treated camel milk was represented in the Tables 4 and 5 respectively.

It was observed that the proteolytic activity of 09 in camel milk was significantly ($p < 0.05$) increased with the incubation periods (0, 3, 6, 9 and 12 h) (Table 4). The proteolytic activity was significantly higher at the 12 h {(0.915) = periodic mean} of incubation for all the inoculation rates (1.0%, 1.5% or 2.0%) than 0 h (0.573), 3 h (0.629), 6 h (0.727) and 9 h (0.854). The proteolytic activity was found highest at 12 h (1.025) of incubation and 2% of inoculation than 1 and 1.5% inoculation. Overall

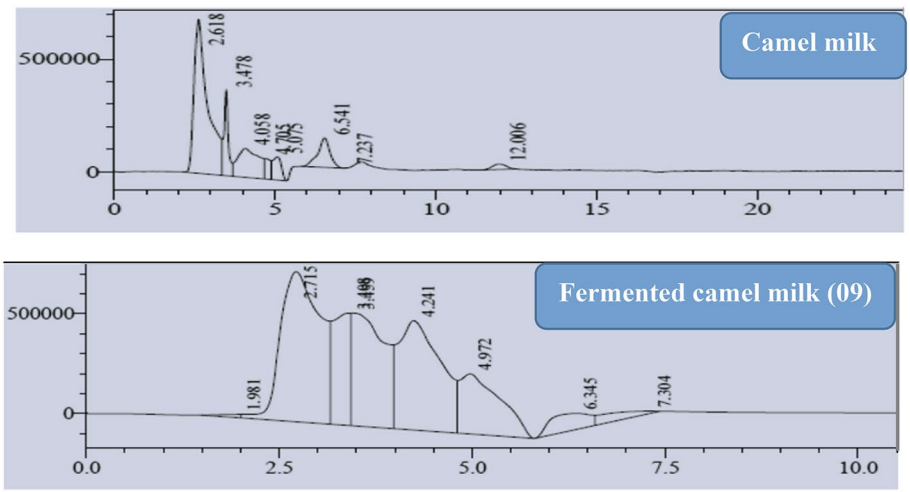

Fig. 1 RP-HPLC chromotogram of Pepticides produced by 09 from fermented camel milk

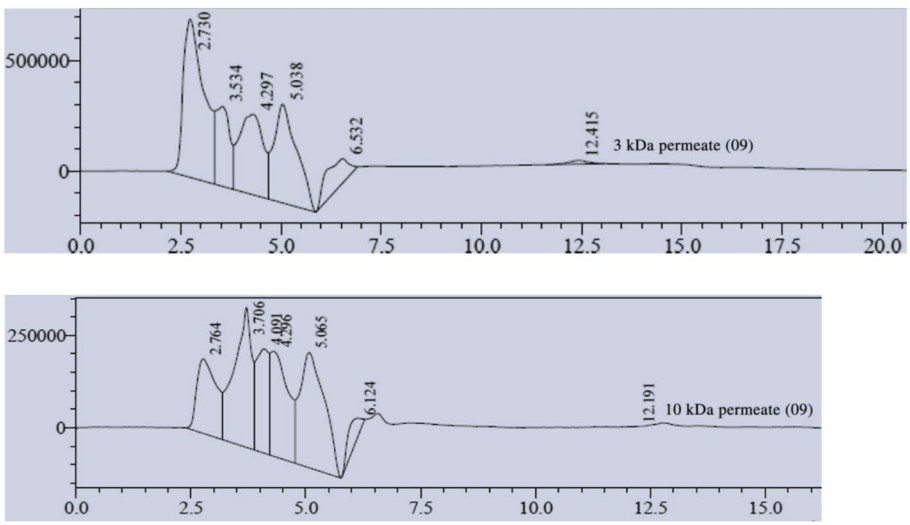

Fig. 2 RP-HPLC chromotogram of 3 and 10 kDa permeate of 09 lactic culture

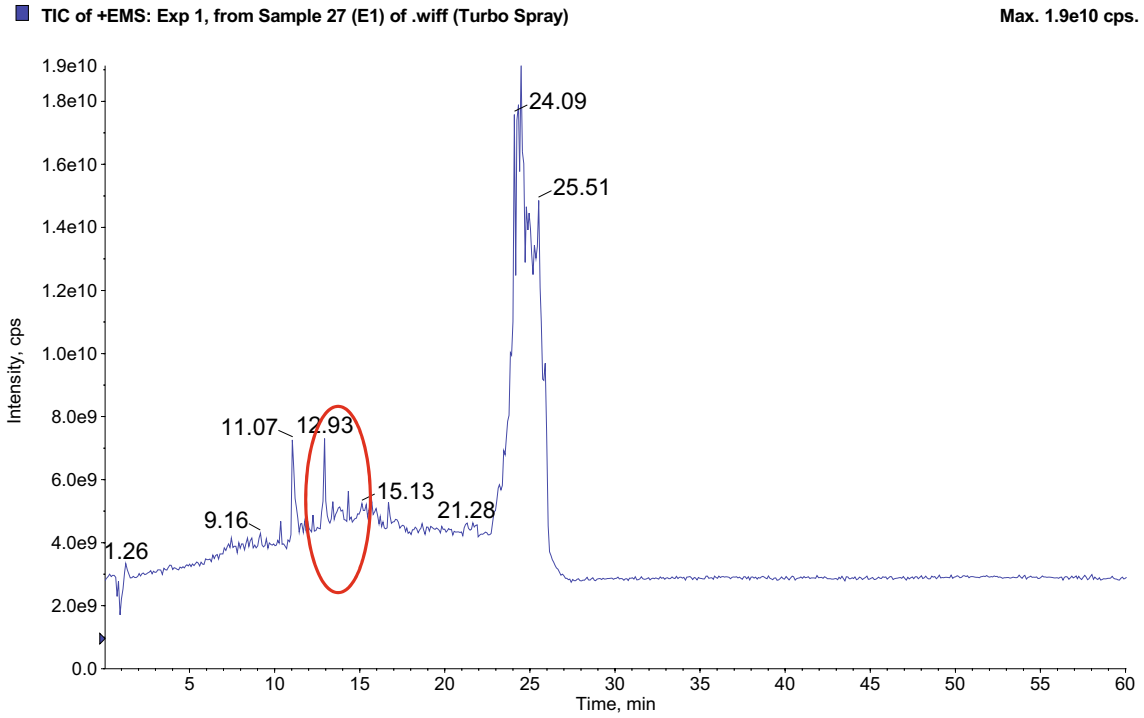

Fig. 3 The total ion chromatogram of fraction E1 of lactic culture LBF generated by EMS to EPI scan in LC-MS

Table 7 Amino acid sequence of peptides (3 kDa Permeate of fermented camel milk) search against online NCBI database

Culture	Fract.	Sequence	Protein/protein precursor	Amino acids
09	H	MDTIEP**VS**VACIS	(VS) Alpha- lactalbumin	f(42–43)
	I	LQYGPLA**DI**LGE	(DI) Alpha-lactalbumin	f(74–75)
LBF	B	C**ISSS**TPP**YD**LNRFK	(ISSS) Beta-CN; Precursor	f(31–34)
			(YD) Alpha-lactalbumin	f(36–37)

Here, Fact. = Fraction (3 kDa) of permeate, Protein/Protein precursor = Those sequences of camel milk protein found matched, Amino acid = fragment of camel milk protein at which query sequence matched, Amino acid = amino acid fraction which matched with query sequence

Table 8 Amino acid sequence of peptides (10 kDa permeate of fermented camel milk) search against online NCBI database

Culture	Fract.	Sequence	Protein precursor/protein	Amino acids
09	Y	ATVQGGIMYR**MP**	(MP) alpha-S_1-CN; precursor	f(226–227)
	A1	GF**KDLLK**GAAKALVKTVLF	(KD_LK) alpha-S_1-CN; precursor	f(99–103)
	B1	ATVQGGIAYR**MP**	(MP) alpha S_1-CN	f(226–227)
LBF	E1	DA**MMNQAV**RE	(AMPVQAV) beta-CN; precursor	f(200–206)
		QSAPGNE**AIPP**	(AIPP) kappa-CN; precursor	f(119–122)
	I1	AGFVLKGYT**KT**SQ	(KT) kappa-CN; precursor	f(124–125)

Here, Fact. = Fraction (10 kDa) of permeate, Protein/Protein precursor = Those sequences of camel milk protein found matched, Amino acid = fragment of camel milk protein at which query sequence matched, Amino acid = amino acid fraction which matched with query sequence

proteolytic activity through 09 was ranged from 0.558 (1.5% inoculation in 0 h of incubation) to 1.025 (2% inoculation at 12 h of incubation).

It was observed that the peptide production of LBF in camel milk was significantly ($p < 0.05$) increased with the incubation periods (0, 3, 6, 9 and 12 h) and their interaction

Table 9 Amino acid sequence of peptides (3 kDa Permeate of fermented camel milk) matched against PIR database

Culture	(F)	Sequence	Sequence searched	Protein/Protein precursor	Amino acid	Protein AC
09	G	NPQYPPGNVQ	YPP	Alpha-S_1-CN	195–197 34–36 187–189	O97943 K4FHK7 K7DXB9
	H	MDTIEPVSVACIS	–	–	–	–
		PPPGSKSTGT	PPP	Alpha-S_1- CN	15–17	A0A0F6QIH7
		GFFALIPGIE	LIP	Alpha-S_1- CN	134–136	O97943
		GPSSGFFGMR	PSS	Alpha-S_1- CN	206–208 45–47 198–200	O97943 K4FHK7 K7DXB9
		XXLVGXQASD	LVG	Alpha-lactalbumin (Fragment)	9–11	Q8WMP6
	I	ELVASIPR	LVA	Alpha-S_1-CN precursor	9–11	O97943
			LVA	Beta- CN	9–11	M1E4K4
			IPR	Alpha-S_1-CN	135–137	K7DXB9
		MIQAEKNPPL	PPL	B-casein (Fragment) Beta-casein precursor, Beta-casein	29–31 93–95	Q28229 Q9TVD0, M1E4K4
		TSIVIIGGGPGGYEAA	–	–	–	–
		LQYGPLADILGE	LQY	Alpha-S_2- CN precursor	87–89	O97944
		QNVLDFHR	–	–	–	–
		MPRKGPAPK	–	–	–	–
	J	VPPAVWNSGNYNS	–	–	–	–
		VNPNTPIR	–	–	–	–
		GANDFMRF	–	–	–	-
LBF	E	DPPFAPRM	DPP	Kappa-CN/Kappa-CN precursor, K-CN (Fragment)	103–105 74–76	L0P3Z7/P79139 W0K8B9
		QNPLDFHR	–	–	–	–
		GFFALIPGIE	LIP	Alpha-S_1-CN precursor/Alpha-S_1-CN/Alpha-S_1-CN precursor	134–136	O97943/K7DXB9/ O97943-2
		VNPNTPIR	–	–	–	–
	A	ITCLSDINSK	–	–	–	–
	B	CISSSTPPYDLNRFK	ISSS	Beta-casein/Beta-casein precursor	31–34	M1E4K4/Q9TVD0
			SSS	Alpha-S_1-CN precursor/Alpha-S_1-CN/ Alpha-S_1-CN precursor Beta-casein, Alpha-S_2-casein precursor	85–87 86–88 32–34 23–25	O97943/K7DXB9/ O97943-2 M1E4K4, O97944

Here, (F) = Fraction of 3 kDa permeate sample, Sequence searched = Tri/ tetra peptides sequence searched, Protein/Protein precursor = Camel milk protein with which query sequence matched, Protein AC = Database ID which represent information about sequence depositor

with different inoculation rates were found significant (Table 5).

The proteolytic activity was significantly higher at the 12 h {(0.928) = periodic mean} of incubation for all the inoculation rates (1, 1.5 and 2%) than 0 h (0.570), 3 h (0.603), 6 h (0.679) and 9 h (0.776). The proteolytic activity was found highest at 12 h (1.023) of incubation and 2% of inoculation as compared to 1 and 1.5% inoculation. Overall proteolytic activity of LBF was ranged from 0.542 (1.0% inoculation in oh of incubation) to 1.023 (2% inoculation in 12 h of incubation).

From the above Tables 4 and 5, it was found that both the cultures exhibited proteolytic activity differently. It differs with different inoculation rates and incubation periods. Increase in proteolytic activity with the different incubation periods was directly related to the amount amino acids required by the lactic cultures during their growth phases based upon which the release of free NH3 groups differs with the inoculation rates. It was reported that the extent of proteolysis varied among strains examined and showed to be the time and strain dependent (Donkor et al. 2007). This report had a similar observation from our study.

In another study, Hati et al. (2015) studied the proteolytic activity of *Lactobacillus rhamnosus* (NS4 and NS6), *Lactobacillus helveticus* MTCC 5463 (V3), *Lactobacillus delbruckii* (09), *Enterococcus feacalis* (ND3), *Enterococcus*

Table 10 Amino acid sequence of peptides (10 kDa permeate) matched against online PIR database

Culture	(F)	Sequence	Sequence searched	Protein/protein precursor	Amino acid	Protein AC
09	Y	ATVQGGIMYRMP	GGI	Alpha-lactalbumin	19–21	P00710
		VPPAVWNSGNYNS	PAV	Beta-casein (Fragment), Kappa-casein/Kappa-casein precursor	4–6/33–35	A0A077B5B2/Q28229
				Beta-casein/Beta-casein precursor	150–152 97–99	L0P3Z7/P79139 M1E4K4/Q9TVD0
				K-casein (Fragment)	121–123	W0K8B9
	Z	MAREHSKENTR	ARE	Beta-casein/Beta-casein precursor	15–17	M1E4K4/Q9TVD0
	B1	ATVQGGIAYRMP	GGI	Alpha-lactalbumin	19–21	P00710
	C1	PPPGSKSTGT	PPP	Alpha-s1-casein (Fragment)	15–17	A0A0F6QIH7
	D1	VIAGGSAAIIG	VIA	Kappa-casein/Kappa-casein precursor	156–158	L0P3Z7/P79139
				Bbeta-casein/Beta-casein precursor	230–232	M1E4K4/Q9TVD0
				K-casein (Fragment)	127–129	W0K8B9
LBF	E1	DAMMNQAVRE	QAV	Beta-casein (Fragment)	111–113	A0A077B5B2
				Beta-casein/Beta-casein precursor	204–206	M1E4K4/Q9TVD0
			AVR	Alpha-S_2-casein precursor	59–61	O97944
	F1	GPSSGFFGMR	PSS	Alpha-S1-casein precursor,	206–208	O97943, K4FHK7
				Alpha-s1-casein (Fragment)	45–47	
				Alpha-s1-casein/Alpha-S1-casein precursor	198–200	K7DXB9/O97943-2
	G1	PPPGSKTSTGT	PPP	Alpha-S_{1-CN} (Fragment)	15–17	A0A0F6QIH7
	H1	RIVLVGPPGAGKGTQAAY-LAQNLSIPHIATGDLFR	LVG	Alpha-lactalbumin (Fragment)	9–11	Q8WMP6/U5YV34
	I1	ELLSEINR	LLS	Beta-casein (Fragment),	85–87	A0A077B5B2
				Beta-casein	178–180	M1E4K4
				B-casein (Fragment)	114–116	Q28229
	J1	VIAGGSAAIIG	VIA	Kappa-casein/Kappa-casein precursor	156–158	L0P3Z7/P79139
				Beta-casein/Beta-casein precursor	230–232	M1E4K4/Q9TVD0
				K-casein (Fragment)	127–129	W0K8B9

Here, (F) = Fraction of 10 kDa permeate sample, Sequence searched = Tri/tetra peptides sequence searched, Protein/Protein precursor = Camel milk protein with which query sequence matched, Protein AC = Database ID which represent information about sequence depositor

feacalis (ND11), *Lactobacillus rhamnosus* (SH8) and *Lactobacillus rhamnossus* (I4) by growing in skim milk @ of 1% 37 °C for 24 h. They found that, NS4 liberated highest amount of amino acids after 24 h of fermentation at 37 °C, while 09 and ND3 also efficiently released amino acids during fermentation. However, V3, NS6, SH8, ND11 and I4 showed comparatively lower proteolytic activity. So, they also concluded that, NS4, 09 and ND3 having strong proteolytic system and maximum proteolytic enzymes producing ability in skim milk medium compared to the other isolates.

Similarly, Rahman et al. (2009) studied the changes in proteolytic activities of *L. acidophilus, L. bulgaricus, L. lactis, St. thermophilus* and mixed cultures of *L. bulgaricus* and *St. thermophilus* (1:1) during fermentation of camel milk. The proteolytic activities exhibited by different cultures were observed after 0, 1.5, 3, 4.5 and 6 h of incubation at 43 °C. They compared the proteolytic activity after 1.5 h of incubation with the 6 h of incubation and found significant increase in proteolysis as similar to our study. In general these results showed that Lactobacillus strains had higher proteolytic activity than the *Lactococcus lactis* strain (Rahman et al. 2009).

Donkor et al. (2007) studied the proteolyitc activity of *L. acidophilus* (L10), *Bifidobacterium* (B94), *L. casei* (L26), *S. thermophilus* (St 1342), *L. delbrueckii* ssp. *bulgaricus* (Lb 1466), *L. acidophilus* (La 4962), *Bifidobacterium* (Bl 536) and *L. casei* (Lc 279) by growing in RSM @ of 1% and providing incubation period of 0, 6, 12, and 24 h at 42 °C. They found that, the amount of liberated amino groups and peptides increased only slightly during fermentation from 0 to 12 h for some strains (*L. acidophilus* L10,

L. acidophilus La 4962, *B. lactis* B94, *B. longum* Bl 536, *L. casei* L26, and *L. casei* Lc 279) but increased significantly ($p < 0.05$) for all strains from 12 to 24 h. They reported that, *Lactobacillus casei* L26 showed the highest proteolytic activity followed in order by *L. delbrueckii* ssp. *bulgaricus* Lb 1466, *S. thermophilus* St 1342 and *L. acidophilus* (La 4962 and L10), *Bifidobacterium* and *L. casei* (Lc 279) with the activity apparently strain specific ($p < 0.05$). Similar to above study, *L. delbrueckii* ssp. *bulgaricus* (09) strain from our study exhibited strong proteolytic activity after 12 h of incubation with the 2% inoculation rate.

Peptide Production (% Relative Proteolytic Activity) Through RP-HPLC

The Relative proteolytic activity was determined according to the method of Vasiljevic and Jelen (2002). Sample was prepared after inoculating in heat treated camel milk at the rate of 2% and incubated at 37 °C for 12 h. The supernatant from fermented sample was injected in RP-HPLC and the change of peptide profile was determined by integrating area under peaks obtained in unfermented camel milk and the fermented camel milk and expressed as R*pa* %.

It was observed that 09 exhibited highest peptide production (47.50%) than LBF (41.81%) (Table 6). Figure 1 represent the RP-HPLC chromatogram of control (unfermented camel milk) and fermented camel milk (09) under optimized growth conditions. And Fig. 2 represent the RP-HPLC chromatogram of 3 and 10 kDa permeate of 09 lactic culture under optimized growth conditions. A comparison of lactic culture's ability for hydrolyzing milk protein was shown by recording area counts from the peptides chromatographic profiles. Peptides produced by the 09 were maximum compared to control milk peptides (unfermented camel milk). Similarly, peptides produced by LBF was found maximum as compared to control milk. Small fractions of peptides were exhibited in control due to some native proteolytic enzymes (Hati et al. 2015). Relative proteolytic activity (%peptide production) of fermented camel milk under optimized growth conditions was presented in the Fig. 1.

Amino Acid Characterization of ACE-Inhibitory Peptides Through LC-MS Analysis

Fractionation of 3 and 10 kDa permeates of fermented camel milk was carried out through RP-HPLC. Each of the sample was injected to collect different fractions eluted at different time and these fractions were used for the characterization of amino acid sequence of peptides through RP-LC/MS. For liquid chromatography, Ekspert ultra LC 100 (Eksigent, USA) was used in conjunction with ABSCIEX QTRAP® 4500 ion trap mass spectrometer via Electron Spray Ionization (ESI) interface with data acquisition and processing software Analyst (version 1.6.1). In built MASCOT script was used to characterize the unknown peptides. The Information dependent analysis (IDA) was optimized with Enhanced Mass Spectra scan (EMS) followed by three Enhanced Product Ion (EPI) scan to identify the unknown peptides. Fragmentation patterns so generated were collected and processed through Analyst software of ABSCIEX. For undigested HPLC fractions the search parameters were set as no enzymatic cleavage with zero mismatches. In addition, search parameters were modified as 1.2 Da for peptide and 0.6 Da product ions mass tolerance, and fixed modification of cysteine to carbamidomethylation and variable modification was set to carboxymethylation and methionine oxidation. Peptides with Mascot Score exceeding the threshold value corresponding to <5% false positive rate, were considered to be positively identified. Further, peptides sequence with significant score was exposed to homology search using BLAST/P online tool (NCBI) against UniProtKB/Swiss-Prot database with taxonomy ID 9838 of *Camelus dromedarius*. Different di/tri and tetra peptides were found matched in PIR database (Barker et al. 2001). Protein Information Resource (PIR) which is popularly used to match di, tri and tetra peptide sequences was used in present study (Tagliazucchi et al. 2016). As it has been reported by many worker that di and tri peptide are the good candidate for ACE-inhibitory activity (Liu et al. 2014; Wu et al. 2006; Hellberg et al. 1991; Cheung et al. 1983). In addition, di, tri-peptide and tetra peptides were searched in Database of Antihypertensive peptides (AHTPDB) which is an ideal platform identification of large number of antihypertensive peptides. AHTPDB provides information about 5978 peptides, out of which 3364 are with IC_{50} value provided and total number of unique antihypertensive peptides was 1694. Hence, it is comprehensive platform for large antihypertensive peptides with all the relevant information associated with them e.g. sequence, source, IC_{50}, pIC50, molecular mass, purification method and therapeutic value etc. AHTPDB also provides information of bitterness/toxicity of the peptides, this is important as many antihypertensive agents have been discovered and used as food additives e.g. FDA has approved 'VPP' & 'IPP' as antihypertensive food additives. If the given peptides have been known with their bitterness/toxicity value, it would be of great use for identification of all those antihypertensive peptides which can be exploited as food additives (Kumar et al. 2015). In this context, VPP was observed in fraction J of 09 culture of 3 kDa permeates and in Y fraction of 10 kDa permeates. Similarly, IPP was observed in LBF fraction E1 of 10 kDa permeate (Tables 11, 12). A total 21 peptides were obtained from 7 fractions of 3 kDa permeate milk samples of two lactic cultures. While 26 peptides were identified from 12 fractions

Table 11 Amino acid sequence of peptides (3 kDa permeate of fermented camel milk) matched against AHTPDB database

Fraction	Sequence	Sequence searched	Pmid/link	Database sequence	Source match with ACE-inhibitory activity
G(09)	NPQYPPGNVQ	PQY	22,249,830	YQKFPQY	Milk proteins
			16,899,666	YQKFPQY	Milk–cheese (goat milk protein and cheeses)
			5555_link	FALPQYLK	Casein derived
		PPG	215,231	GPPGAP	Bovine (*Bos taurus*)
			6977_link	IPPGVPYWT	Soybean (*Glycine max*)
		GNV	2933_link	TGGGNV	Fish skin
			21,945,677	TGGGNV	Fish (Pacific cod skin)
		EPV	3627_link	YQEPVLGPVRGPFPIIV	Cheese [Cheddar (with probiotics)]
			3635_link	YQEPVLGPVRGPF	Cheese (Fresco)
			3636_link	YQEPVLGPVRGPFPI	Cheese (Fresco)
H(09)	MDTIEPVSVACIS	IEP	2000_link	IEPQG	Oat proteins
			2196_link	IEP	Milk
		SVA	21,773,582	ALKAWSVAR	Bovine whey proteins
			10,399,349	ALKAWSVAR	Whey protein
	PPPGSKSTGT	PPP	2888_link	GPPP	Fish (catfish muscle)
			5755_link	VHLPPP	Cereals [maize (*Zea mays*)]
		PPG	23,598,136	LIPPGVPY	Wine
			18,471,845	JKWPPGKVPP	Snake (*Bothrops moojeni*)
			6977_link	IPPGVPYWT	Soybean (*Glycine max*)
			1984_link	AFPGS	Soybean proteins
			2178_link	LQPGS	Fish
	GFFALIPGIE	GFF	20,941,517	GFF	ND
			23,871,047	GFF	ND
		ALI	4617_link	QNALIVRYTR	Milk [sheep raw milk cheese (synthesised)]
		LIP	23,598,136	LIPPGVPY	Wine
			23,194,537	LIPPGVPY	ND
		IPG	18,808,143	GAPGPAGPGGIPGERG	Chicken (*Gallus gallus*)
			18,808,143	GIPGERGPVGPSG	Chicken (*Gallus gallus*)
H(09)	GFFALIPGIE	PGI	20,151,679	MYPGIA	Pork (*Sus domesticus*)
			20,941,517	PGI	ND
	GPSSGFFGMR	GPS	24,215,325	GPSMR	Fungi [mushroom (*P.Cystidiosus*)]
			18,808,143	GIPGERGPVGPSG	Chicken (*Gallus gallus*)
			24,915,368	GAVGPSG	Chicken leg bone
	XXLVGXQASD	VG	10,717,843	VGINYWLAHK	Whey protein
			16,162,521	LNVVGETVE	Milk (caprine kefir)

Table 11 (continued)

Fraction	Sequence	Sequence searched	Pmid/link	Database sequence	Source match with ACE-inhibitory activity
I(09)	ELVASIPR	LVA	4619_link	EGPKLVAS	Milk [sheep raw milk cheese (synthesised)]
			10,717,843	YGLVAGTW	Whey protein
	MIQAEKNPPL	IQA	1183_link	RDMPIQAF	Milk
			17,483,275	VPYPQRDMPIQA	Milk (bovine sodium caseinate)
		EKN	5833_link	TKKTKLTEEEKNRL	Milk (bovine α-casein)
		NPP	YTD	LLNPPHQIYP	Casein (Beta casein)
		PPL	19,524,628	PPL	Milk
			1799_link	VYPFPGPIHNSLPQNIP-PLTQT	Casein
			1165_link	LPQNIPPLTQTPVVVPP-FLQPEVMGVSK	Milk
			3647_link	NIPPLTQTPV	Milk (fermented milk)
	TSIVIIGGGPGGYEAA	IVI	23,871,047	IVIF	ND
		VII	3711_link	VIIF	Cuttlefish skin gelatin
		IGG	24,915,368	IGGSI	ND
			17,117,396	PGG	ND
		GGY	1137_link	GGY	Milk
		EAA	24,915,368	WPEAAELMMEVDP	Fish (tuna dark muscle)
		GGP	24,915,368	EGGPKP	Chicken leg bone
		YEA	23,598,136	YEAP	Soybean whey proteins
	LQYGPLADILGE	LQY	2101_link	LQY	Milk derived
			5839_link	FPQYLQY	Milk (bovine casein)
		YGP	23,271,625	VECYGPNRPQF	Algae
		PLA	5751_link	IGDEPLANYL	Jellyfish *Stomolophus nomurai*
	QNVLDFHR	NVL	24,380,081	TTENVLFG	Fungi (mushroom)
I(09)	MPRKGPAPK	GPA	2253_link	GPA	Milk derived
			23,194,537	GPAGAPGAA	ND
J(09)	VPPAVWNSGNYNS	VPPA	23,271,625	VVPPA	Algae (*Chlorella vulgaris*)
		VPP	2009_link	VPP	Milk (fermented milk)
	VNPNTPIR	–	–	–	–
	GANDFMRF	–	–	–	–
E(LBF)	DPPFAPRM	DPP	2876_link	VWDPPKFD	Cuttlefish muscle
	QNPLDFHR	–	–	–	–
	GFFALIPGIE	GFF	20,941,517	GFF	ND
		LIP	23,598,136	LIPPGVPY	Wine
	VNPNTPIR	PIR	6447_link	AVNPIR	Cereals (maize endosperm)
A(LBF)	ITCLSDINSK	ITC	2096_link	ITC	Milk derived
B(LBF)	CISSSTPPYDLNRFK	PPY	8,829,536	LLPPY	Cereals [Maize (*Zea mays*)]
		ISSS	2070_link	ISSSK	Soybean protein

PMID/LINK it is online link of the already reported source of the peptide sequence

Sequence peptide sequences which were characterized from fermented camel milk (3 kDa permeates)

Sequence searched different di/tri/tetra peptide sequences from the query sequences which were searched against AHTPDB

Database sequence peptide sequences which were reported already and found match with our peptide sequences

Source match with ACE-inhibitory activity ACE-inhibitory peptide sources from database

of 10 kDa permeates. In case of 3 kDa permeates, maximum fractions 4 were collected from 09 fermented camel milk using RP-HPLC while in case of 10 kDa permeates, maximum 16 fractions were collected from lactic culture LBF fermented camel milk. Total ion chromatogram of fraction E1 of 10 kDa permeate of LBF culture generated by EMS to EPI scan in LC-MS was presented in the Fig. 3. Presence of similar sequences such as PPPGSKSSTGT in

Table 12 Amino acid sequence of peptides (10 kDa Permeate) matched against AHTPDB database

Y(09)	ATVQGGIMYRMP	GGI	5752_link	FGGIDDINQIGQSD	Jellyfish *Stomolophus nomurai*
		VQG	23,598,136	LVQGS	Soybean (Fermented Soybean)
		RMP	2003_link	VRMPQ	Cereals (Barley protein B-Hordein)
Z(09)	APGQDFMRF	APG	23,271,625	IAPG	Algae (*Spirulina platensis*)
	MAREHSKENTR	–	–	–	–
	AVHGQFATL	ATL	1786_link	AVESTVATL	Casein
A1(09)	GFKDLLKGAAKALVKTVLF	MP	18,211,015	MP	Prince-of-Wales feather (*Amaranthus hypochondriacus*)
			24,915,368	MPF	Egg
		LLK	16,162,521	QLLKLK	Milk (caprine kefir)
		GAA	22,249,830	GAAELPCSADWW	Bullfrog muscle protein hydrolysate
B1(09)	ATVQGGIAYRMP	MP	18,211,015	MP	Prince-of-Wales feather (*Amaranthus hypochondriacus*)
		GGI	5752_link	FGGIDDINQIGQSD	Jellyfish *Stomolophus nomurai*
		IAY	24,915,368	IAYKPAG	Spinach Rubisco
C1(09)	PPPGSKSTGT	PPG	17,117,396	PPG	ND
		PPP	17,117,396	PPP	ND
		PGS	1984_link	AFPGS	Soybean proteins
D1(09)	VRTPVTVQTKVDNIKKY	VRTP	5923_link	VRTPE	Milk (caprine lactoglobulin)
	VIAGGSAAIIG	GGS	24,915,368	IGGSI	ND
		VI	17,117,396	VI	ND
		IG	6,243,277	IG	ND
E1(LBF)	DAMMNQAVRE	AVR	1797_link	INNQFLPYPYYAK-PAAVR	Casein
		VRE	6554_link	KVREGT	Egg (Hen ovotransferrin)
	QSAPGNEAIPP	AIPP	22,249,830	MAIPPKK	Milk proteins
			4298_link	MAIPPK	Milk-Cheese (Goat milk protein and cheeses)
		IPP	21,185,549	IPP	Fermentation with *L. helveticus* and *S. cerevisiae*
			2008_link	IPP	Milk (Fermented Milk)
			2243_link	IPP	Milk derived
F1(LBF)	VNPNTPIR	PIR	6447_link	AVNPIR	Cereals (Maize endosperm)
	QNVLDFHR	NVL	24,380,081	TTENVLFG	Fungi (Mushroom)
	GPSSGFFGMR	PSS	2219_link	LQPSS	Milk
			24,262,574	PSSNK	Fungi (*Agaricus bisporus*)
		GFF	20,941,517	GFF	ND

Table 12 (continued)

G1(LBF)	PPPGSKSTGT	PPP	23,194,537	SPPPFYL	ND
G1(LBF)	PPPGSKSTGT	PPP	2888_link	GPPP	Fish (Catfish muscle)
			5755_link	VHLPPP	Cereals (Maize (*Zea mays*))
		PGS	1984_link	AFPGS	Soybean proteins
		GT	3650_link	GTW	Milk (Fermented Milk)
	SRPYSFGL	RPY	7,763,772	YRPY	Fish (Bonito (Sarda bowel autolysate))
			5308_link	YRPY	ND
		GL	1138_link	GLDIQK	Milk
	SMIGGVMSKG	IGG	24,915,368	IGGSI	ND
H1(LBF)	RIVLVGPPGAGKGTQAAY-LAQNLSIPHIATGDLFR	LFR	6647_link	LFRQ	ND
			24,380,081	RLSGQTIEVTSEYL-FRH	Fungi (Mushroom (*Pholiota adiposa*))
		LFR	23,140,680	RLSGQTIEVTSEYL-FRH	Fungi (Mushroom (Branched Oyster Mushroom (*Pleurotus cornucopiae*))
H1(LBF)	AVHGQFATL	ATL	1786_link	AVESTVATL	Casein
I1(LBF)	ELLSEINR	ELL	1,368,548	PVQALLLNQELLLNP	Milk (human β casein)
	AGFVLKGYTKTSQ	AGF	24,915,368	VKAGF	Porcine myosin B
		SQ	8,829,536	FSQ	Cereals (Maize (*Zea mays*))
			23,845,432	FTESQS	Milk (Bovine skim milk)
	QNVLDFHR	NVL	24,380,081	TTENVLFG	Fungi (Mushroom)
J1(LBF)	SPPFAPRL	SPP	5960_link	SPPEIN	Milk (fermented milk)
J1(LBF)	SPPFAPRL	SPP	2283_link	SPP	Milk derived
			23,194,537	SPPPFYL	ND
		PPF	2292_link	PPF	Milk derived
	MDFNISGIGNVS	MDF	23,598,136	MDFLI	Chickpea (legumin fraction)
	VIAGGSAAIIG	GGS	24,915,368	IGGSI	ND
		VI	17,117,396	VI	ND
		IG	6,243,277	IG	ND

Here, PMID/LINK = It is online link of the already reported source of the peptide sequence

Sequence = Peptide sequences which were characterized from fermented camel milk (10 kDa permeates)

Source match with ACE-inhibitory activity = ACE-inhibitory peptide sources from database

Sequence searched = Different di/tri/tetra peptide sequences from the query sequences which were searched against AHTPDB

Database sequence = Peptide sequences which were reported already and found match with our peptide sequences

09 and LBF and QNVLDFHR in 09 and LBF of different fractions revealed cultures may have similar kind of proteolytic activity of among the lactic cultures. The finding was reported by previous worker (Rodríguez-Figueroa et al. 2012). All the characterized sequences from 3 and 10 kDa fractions were matched against NCBI (Database) using BLAST/P (Tables 7, 8), PIR database (Tables 9, 10) to confirm camel milk protein and matched in AHTPDB database (Tables 11, 12 to confirm ACE-inhibitory activity of characterized peptides. Presence of similar sequences such as

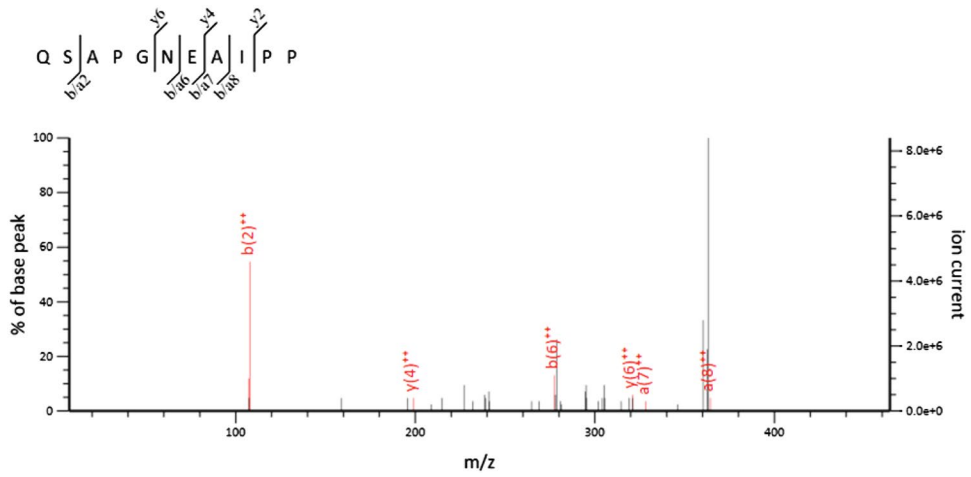

Fig. 4 MS/MS spectrum of fraction E1 inspected in MASCOT database. Identified as QSAPGNEAIPP with expected and calculated molar mass 1080.3178 and 1079.5247 respectively

Table 13 Molecular weight of different identified peptides from fermented camel milk

3 kDa permeate	Sample	Sequence	Expected mol. mass	Calculated mol. mass	Observed mol. mass
09	G	NPQYPPGNVQ	1112.5334	1112.5251	557.2740
	H	MDTIEPVSVACIS	1436.9182	1437.6367	479.9800
		PPPGSKSTGT	927.1082	927.4662	310.0434
		GFFALIPGIE	1062.3912	1062.5750	355.1377
		GPSSGFFGMR	1056.3618	1057.4651	353.1279
		XXLVGXQASD	1020.7582	1021.3392	341.2600
	I	ELVASIPR	884.4304	883.5127	295.8174
		MIQAEKNPPL	1140.2499	1139.6008	286.0697
		TSIVIIGGGPGGYEAA	1461.2318	1460.7511	731.6232
		LQYGPLADILGE	1288.7059	1287.6711	323.1837
		QNVLDFHR	1027.3582	1027.5199	343.4600
		MPRKGPAPK	996.9615	996.5538	333.3278
	J	VPPAVWNSGNYNS	1404.6128	1403.6470	469.2115
		VNPNTPIR	909.7294	909.5032	304.2504
		GANDFMRF	955.5712	956.4174	319.5310
LBF	E	DPPFAPRM	930.0866	929.4429	311.0361
		QNPLDFHR	1024.4182	1025.5043	342.4800
		GFFALIPGIE	1062.5496	1062.5750	355.1905
		VNPNTPIR	909.3982	909.5032	304.1400
	A	ITCLSDINSK	1149.1492	1149.5700	575.5819
	B	CISSSTPPYDLNRFK	1783.2432	1783.8563	595.4217
10 kDa permeate	Sample	Sequence	Expected mol. mass	Calculated mol. mass	Observed mol. mass
09	Y	ATVQGGIMYRMP	1323.5103	1322.6475	662.7624
		VPPAVWNSGNYNS	1404.7818	1403.6470	469.2679
		MAREHSKENTR	1373.7120	1373.6470	458.9113
		MPPPGSKSEGT	1103.4686	1102.4965	552.7416
	Z	APGQDFMRF	1068.5902	1067.4859	357.2040
		MAREHSKENTR	1373.5091	1373.6470	458.8436
		AVHGQFATL	942.9027	942.4923	315.3082
	A1	GFKDLLKGAAKALVKTVLF	2018.2014	2018.2292	673.7411
		LQYGPLAIILGE	1284.9432	1285.7282	429.3217

Table 13 (continued)

10 kDa permeate	Sample	Sequence	Expected mol. mass	Calculated mol. mass	Observed mol. mass
	B1	ATVQGGIAYRMP	1323.7143	1322.6289	662.8644
		AEKLEPVLPLIV	1320.3783	1319.8064	441.1334
		GPSGFLGMR	936.6167	936.4487	313.2128
		SRPYSFGL	924.9855	925.4658	309.3358
		KDLMHRDDKT	1272.5547	1273.6085	425.1922
		CSKGGVGRGYGIG	1268.5500	1267.5979	635.2823
		GLVASIPR	810.4067	811.4916	271.1429
	C1	PPPGSKSTGT	927.3411	927.4662	310.1210
		AVHGQFATL	942.5737	942.4923	315.1985
		GLVASIPR	810.3864	811.4916	271.1361
		SPPFAPRL	884.3182	883.4916	295.7800
		QPSQDFMRF	1171.3527	1170.5128	391.4582
	D1	VRTPVTVQTKVDNIKKY	1987.0850	1988.1419	663.3690
		VIAGGSAAIIG	927.7753	927.5389	310.2657
		GPSSGFFGMR	1056.3262	1057.4651	353.1160
		AVHGQFATL	942.5983	942.4923	315.2067
		LQYGPLAYILGE	1336.7462	1335.7074	335.1938
		IQAEKNPRL	1068.3336	1067.6087	357.1185
		VNANTPIR	882.4604	883.4875	295.1607
LBF	E1	DAMMNQAVRE	1195.2915	1195.4961	399.4378
		QSAPGNEAIPP	1080.3178	1079.5247	361.1132
	F1	VNPNTPIR	909.7294	909.5032	304.2504
		VNANTPIR	884.4225	883.4875	295.8148
		DVVSPPVCGN	1044.4866	1043.4594	349.1695
		QNVLDFHR	1027.1781	1027.5199	343.4000
		MPRKGPAPK	996.5153	996.5538	333.1790
		GPSSGFFGMR	1056.5286	1057.4651	353.1835
		APGQDFMRF	1068.2946	1067.4859	357.1055
	G1	PPPGSKSTGT	927.7048	927.4662	310.2422
		SRPYSFGL	924.7034	925.4658	309.2417
		SMIGGVMSKG	966.4128	965.4674	323.1449
	H1	RIVLVGPPGAGKGTQAAY-LAQNLSIPHIATGDLFR	3601.9048	3600.9893	1201.6422
		AVHGQFATL	942.5912	942.4923	315.2043
		APGQDFMRF	1068.3800	1067.4859	357.1339
		APSGFMGMR	967.2382	968.4208	323.4200
	I1	ELLSEINR	972.6818	972.5240	325.2345
		AGFVLKGYTKTSQ	1398.3891	1398.7507	467.1370
		QNVLDFHR	1026.5678	1027.5199	343.1965
		MIYSTEVVNMNM	1478.6661	1478.6091	493.8960
		QPSQDFMRF	1171.2389	1170.5128	391.4202
	J1	SPPFAPRL	884.6827	883.4916	295.9015
		MDFNISGIGNVS	1267.5141	1268.5707	634.7643
		VIAGGSAAIIG	927.5127	927.5389	310.1782

PPPGSKSSTGT and QNVLDFHR in 09 and LBF of different fractions revealed cultures may have similar kind of proteolytic activity of among the lactic cultures. The finding was reported by previous worker (Rodríguez-Figueroa et al. 2012). Interestingly, peptide fraction E1 obtained from LBF (*Lactobacillus fermentum*) showed amino acid sequence QSAPGNEAIPP (Fig. 3) with Pro in the C-terminal position which reflect ACE-inhibitory activity. This finding was supported by the investigation of Pripp et al. (2004) who also specified the relationship between hydrophobicity and positively charged amino acids in the C-terminal position for ACE-inhibitory activity.

Highlighted Portion in the Chromatogram shows Spectra of QSAPGNEAIPP Peptide from E1 Fraction (10 kDa Permeate) of Lactic Culture LBF

It has been reported that α, β-, and k-CN are precursors of bioactive peptides (Mills et al., 2011). However, in the current work, casein protein as well as whey protein such as α-LA, were found to be important sources of peptides with ACE-inhibitory activity. Camel milk fermented with LBF showed QSAPGNE**AIPP** peptide sequence derived from Kappa-casein (119–122) which have encrypted the hypotensive tripeptide (IPP) reported by Nakamura et al. (1995). MS/MS spectrum of fraction E1 of LBF (3 kDa permeate) was presented in the Fig. 4.

Similarly, peptide sequences of 3 and 10 kDa permeates were found matched with various reported sequences and sources (Tables 11, 12, 13) like milk protein, soy protein, cereals, fungi, algae, fermented milks, cheese, wine, fish and legumes using AHTPDB.

Conclusion

This work evidenced the ACE-inhibitory effect of camel milk fermented with *Lactobacillus bulgaricus* NCDC (09) and *Lactobacillus fermentum* TDS030603 (LBF). These Lactobacillus cultures had shown maximum ACE-inhibitory activity, pepX activity and proteolytic activity during the fermentation of camel milk. Fractionation of the fermented camel milk under optimized growth conditions revealed that peptides involved in the ACE-inhibitory activity are of lower molecular weight (3 and 10 kDa permeates). This observation was confirmed by RP-LC/MS analysis, which led to the identification of many unknown ACE-inhibitory peptides and known peptides such as VPP and IPP from fermented camel milk. Moreover, these findings showed the another source of ACE-inhibitory peptides from "Camel milk (*Camelus dromedarius*)" in India, which has an anti-diabetic property. Preparation of functional foods and validation of ACE-inhibition through clinical studies will add another health claim of fermented camel milk.

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