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Antioxidant and antimicrobial activity of camel milk casein hydrolysates and its fractions

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ABSTRACT

Camel milk casein hydrolysates by Alcalase, α-Chymotrypsin and Papain were ultra-filtered and different fractions were assessed for antioxidant and antimicrobial activity. The casein hydrolysate fractions were analyzed for antioxidant activities viz. 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing antioxidant power assay (FRAP), and antimicrobial activity by inhibition zone assay. In ABTS, DPPH and FRAP assay, the inhibition activity was recorded significantly ($P < 0.05$) higher for whole hydrolysares whereas among fractions F2 (1–5 kDa), F3 (5–10 kDa) of all the three hydrolysates had comperatively higher antioxidant activity. It was also observed that α- Chymotrypsin could produce protein hydrolysates and fractions with higher antioxidant activities. The antimicrobial activity (zone of inhibition; mm) was also recorded higher for whole hydrolysates as compared to their fractions; however, among different fractions the inhibition zones were almost comparable. It was also observed that Alcalase and α-Chymotrypsin could produce paptides with higher antimicrobial activity. The results suggested that camel milk casein hydrolysates could be fractioned to get specific molecular weight peptides, however, for food application or for direct human consumption, use of whole hydrolysates could be more beneficial with regards to its functionalities and cost of production.

1. Introduction

Milk proteins are one of the richest sources of essential amino acids required for growth and maintenance. It also plays an important role in the promotion of health and disease prevention (Meisel, 2005). Intact milk proteins contain array of encrypted peptides that can be released by enzymatic hydrolysis and fermentation. It is well established that endogenous enzyme presented in milk (principally plasmin) liberates from casein peptides that are involved in regulation of milk secretion, milk clotting and mammary gland innate defense system (Silanikove et al., 2006; Leitner et al., 2006, 2011). Recently, these peptides have also been explored for its functionalities as food additives for the formulation of functional foods as well as nutraceutical and pharmaceutical products.

The beneficial effects of food derived bioactive peptides on human health has been documented by many researchers (Haque et al., 2009; Korhonen and Pihlanto, 2006; Mao et al., 2011) but, it was Marcuse (1960) who reported for the first time that peptides derived from dietary proteins have antioxidant activity. Since then, various protein sources viz. casein (Suetsuna et al., 2000; Kumar et al., 2016a), whey proteins, egg proteins (Sakanaka and Tachibana, 2006), fish proteins, muscle protein, plant proteins such as peanut proteins (Hwang et al., 2010), and larval proteins (Wang et al., 2013) have been explored to investigate the antioxidant properties.

Bioactive peptide derived from food protein also shows a broad range of activity against microorganisms of food spoilage and/or health significance. The antimicrobial peptides derived from milk proteins present the great advantage of being produced from harmless and inexpensive sources. Hence, there is a growing interest in the utilization of these bioactive peptides as food grade biopreservatives or as health-promoting food supplements in the food industry. Antimicrobial peptides (AMP) mostly act as bactericidal. All AMPs interact with the cell wall or membrane of bacteria. AMPs

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have an affinity for the anionic phospholipids and lipopolysaccharides found in cell walls and membranes of bacteria (Barzyka et al., 2009). The antimicrobial activity and its machanisms have been explained by many scientists (Deegan et al., 2006; Lopez-Exposito et al., 2007; Umuhumuza et al., 2011).

The differences in milk composition and structure of its protein components of dromedary camel (*Camelus dromedaries*) milk differentiate it from other milk in its functional and biological properties (Kumar et al., 2016b). Out of two milk proteins, casein proteins are the major proteins in camel milk and β-CN constitutes about 65% of total caseins (Kappeler et al., 2003) whereas the whey proteins are present in smaller amount (20–25% of total protein) in which the β-lactglobulin is deficient. Significant therapeutic attributes of camel milk such as anti-cancer and anti-diabetic properties have been suggested by many researchers (Agrawal et al., 2003; Magjeed, 2005), but until recently, the research focused on milk derived peptides were mainly on bovine and to smaller extent on ovine and caprine milk proteins. In the previous experiments, camel casein were hydrolysed by proteolytic enzymes from different sources viz. Alcalase (microbial), α-Chymotrypsin (animal) and Papain (plant) and antioxidant activities of the hydrolysates were reported by Kumar et al. (2016a). Commercial production of bioactive peptides from milk proteins has been limited by a lack of suitable large-scale technologies. However, membrane separation techniques is utilized to separate the peptides with a specific molecular weight range (Korhonen and Pihlanto, 2006). Stepwise ultrafiltration using cut-off membranes of low molecular mass for separating out small peptides from high molecular mass residues and remaining enzymes. Therefore, this study was undertaken to produce peptide fractions by ultrafiltration and to investigate its antioxidant and antimicrobial properties.

2. Materials and methods

2.1. Chemical and reagents

Fine chemicals such as 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH) obtain from Sigma–Aldrich Chemical Co. India and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from MP Biomedicals, India. The dehydrated microbiological medis and other analytical chemicals were procured from reputed companies and used without further purification. The freeze dried cultures of various pathogenic and spoilage organisms' viz. *Escherichia coli* (MTCC No. 2991), *Bacillus cereus* (MTCC No. 6728), *Staphylococcus aureus* (MTCC No. 7443) and *Listeria monocytogenes* (MTCC No. 657) were procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh-160036, India.

2.2. Fractionation of hydrolysed camel casein solutions

The reconstituted casein proteins hydrolysates using different enzymes as reported by Kumar et al. (2016a) were used in this study for ultrafiltration. The casein hydrolystes (with Alcalase: CA, α-Chymotrypsin: CC and Papain: CP) were sequentially ultrafiltered through a Millipore 8400 ultra-filtration unit (Amicon, Millipore, USA) using regenerated cellulose membranes (Diameter 76 mm, Amicon Bioseparations, USA) with different molecular weight (MW) limits (Fig. 1). Briefly, the whole hydrolysates (F0) were first ultra-filtered through a membrane with 10 kDa nominal molecular weight limit (NMWL) under 40 psi nitrogen gas. This process yielded two fractions: retentate (>10 kDa; F4) and permeate (<10 kDa). The permeate was further ultra-filtered through a 5 kDa NMWL membrane to obtain the second retentate (5 and 10 kDa; F3) and permeate (<5 kDa) and subsequently the second permeate was ultra-filtered through a 1 kDa NMWL membrane to yield the third retentate (1 and 5 kDa; F2) and permeate (<1 kDa; F1). All retentates and permeates were stored at −20 °C till further analysis.

2.3. Antioxidant activity assay

2.3.1. 2-2-Azinobis-3ethylbenthiazoline-6-sulphonic acid ($ABTS^+$) radical scavenging activity

The spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined according to method described by Kumar et al. (2016a). ABTS radical cation ($ABTS^+$) was produced by reacting $ABTS^+$ stock solution with equal volume of 2.45 mM potassium persulphate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at room temperature for 16 h before use. Prior to use, the stock solution was diluted with ethanol to an absorbance of 0.70 at t_0 (0 min) and equilibrated at 30 °C exactly 6 min after initial mixing. About 1 ml of $ABTS^+$ working standard solution was mixed with 10 μl of hydrolysate/standard and absorbance was measured after 20 min (t_{20}) at 734 nm in multimode reader (Synergy H1Hybrid Multi-Mode Microplate Reader, BioTek India, Mumbai). The $ABTS^+$ activity was calculated by using formula: ABTS activity (% inhibition) = $[(0.7 - At_{20})/0.7] \times 100$.

2.3.2. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical by added antioxidants in samples was estimated following the method of Brand-Williams et al. (1995) with slight modification. 1 ml of DPPH reagent (100 μM) was mixed with 0.25 ml of 0.1 M Tris–HCl buffer (pH 7.4) and 25 μl of hydrolysate sample in test tubes. The content was gently mixed and the absorbency in time t = 0 min (t_0) was measured at 517 nm using multimode reader (Synergy H1Hybrid Multi-Mode Microplate Reader, Bio Tek India, Mumbai). The sample tubes were also incubated at room temperature under dark for measurement of absorbency in time t = 20 min (t_{20}). Ethanol was used as blank. The free radical scavenging activity was calculated as decrease in absorbance from the equation: Scavenging activity (% inhibition) = $100 - [(At_{20}/At_0) \times 100]$.

2.3.3. Ferric reducing-antioxidant power (FRAP) assay

The FRAP was assessed according to Benzie and Strain (1999) using multimode reader. Briefly, 900 μl of working FRAP reagent (300 mM acetate buffer, pH 3.6: 20 mM ferric chloride solution: 10 mM TPTZ in 40 mM HCl:10:1:1) prepared fresh was mixed with 100 μl of hydrolysate sample and incubated for 20 min 37 °C before recording the absorbance at 593 nm by using multimode reader (Synergy H1Hybrid Multi-Mode Microplate Reader, Bio Tek India, Mumbai). FRAP values were obtained by comparing the absorption change in the test mixture with those obtained from increasing concentrations of Fe^{3+} and expressed as mmol of Fe^{2+} equivalents per mL of sample. Ferrous sulphate was used as standard for standard curve preparation.

2.4. Anti-microbiological activity assay

Four pathogenic and spoilage organisms' viz. *Escherichia coli* (MTCC No. 2991), *Bacillus cereus* (MTCC No. 6728), *Staphylococcus aureus* (MTCC No. 7443) and *Listeria monocytogenes* (MTCC No. 657) were used in assay protocol. The freeze dried cultures were activated and cultures were maintained at refrigeration temperature by sub culturing. The required bacterial population was obtained by serial dilution using sterile peptone water. The dose rate of the inoculums was standardized on the basis of cell number in the inoculums. The dose rate of the above mentioned microbial cultures was optimized in the range of 10^4–10^5 cfu/ml.

Pour plate technique was used for inoculation and media solidification. 1 ml of the test culture (10^4-10^5 cfu/ml) were uni-

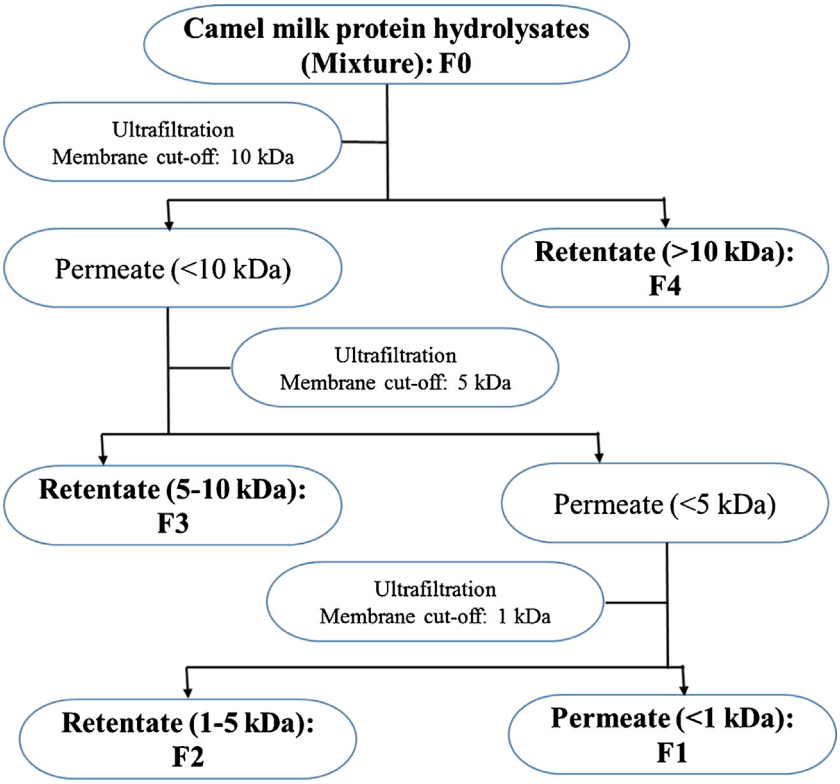

Fig. 1. Flow chart of sequential ultrafiltration protocol of camel milk protein hydrolysates using different membrane cut-off size.

formly distributed by pouring 15–20 ml of pre-sterilized media and allowed for solidification. In each plate three wells (8–10 mm diameter) were made using sterile cork borer. About 100 μl of each hydrolysates and fractions were poured into well onto solid media in nutrient agar for all the test organisms and incubated at 37 °C for 24 h. The diameters of inhibitory zone surrounding the wells were measured using digital vernier calipers.

2.5. Statistical analysis

All the experiments were repeated three times and parameters were analysed in triplicate (n = 9). Data were expressed as means with standard error. Two-way analysis of variance (ANOVA) was done by comparing the means by using Duncan's multiple range test (DMRT), at 95% confidence level using a SPSS package (SPSS 17.0 for Windows, SPSS Inc., USA).

3. Results and discussion

Research efforts were focused on the fractionation of peptides from camel milk casein proteins (CCP) and subsequently different antioxidant and antimicrobial assays were carried out and the data obtained were statistically analysed, presented in tables, and are also discussed in detail in the following sections. The CCP hydrolysates with Alcalase (CA), α-Chymotrypsin (CC) and Papain (CP) were ultra filtered to get the different farctions viz. F0: whole hydrolysates, F1: <1 kDa, F2: 1–5 kDa, F3: 5–10 kDa and F4: >10 kDa. These fractions were *in-vitro* evaluated for antioxidant (ABTS, DPPH and FRAP) and antimicrobial activity (zone inhibition assay). In the current area of study, there are relatively few studies available in the literature, therefore, comparative inference was drawn on discussion with other milk and protein sources.

3.1. Antioxidant activity of CCP hydrolysates and its fractions

The cationic radical scavenging activity of $ABTS^{+}$ is most frequently utilized to measure antioxidant activity of food ingredients and processed meat/food products. Since, the reagents dissolve well in both aqueous hydrophilic and organic solvent hydrophobic groups, this assay measures both the hydrophilic and lipophilic antioxidants. Its efficiency depends upon the number of aromatic rings, nature of hydroxyl groups and molecular weight (Hangerman et al., 1998). Hence, it is required to verify the antioxidant activity of food ingredients by conducting different assays, because the mechanism of action in one assay differs from another and also influences the end results. In this experiment, it was included because the selection of ingredients were aimed for inhibition of lipid oxidation in meat products in further experiments. The results of ABTS (% inhibition) assay of camel milk casein hydrolysates with different enzymes are presented in Table 1. Among fractions and whole hydrolyastes of the group CA, the highest activity was recorded for F0 (79.69 ± 0.10) followed by F3, F2, F4 and F1. Although, the ABTS activity of F4 was comparable to F1 and F2, the other values were significantly ($P < 0.05$) varied from each other. Among the group CC, the fraction F0 recorded highest ABTS activity (91.28 ± 0.13) followed by F3, F2, F4 and F1 (81.09 ± 0.18). The ABTS activity of F1 and F4 were comparable, but the other groups differed significantly ($P < 0.05$) from each other. For the group CP, the ABTS activity was recorded lower as compares to other two groups, but among different fractions of the same group, the highest activity was again recorded for F0 (70.90 ± 0.14), followed by F3, F2, F1 and F4 (62.24 ± 0.22). In the group CP, the ABTS activity of F1 was comparable to that of F2 and F4, but the other two fractions differed significantly ($P < 0.05$). Among the three groups i.e, CA, CC and CP, a significantly higher ABTS activity was recorded for the group CC for all the fractions. The fraction F0 i.e, whole hydrolysate exerted

Table 1
Antioxidant activity of camel casein protein (CCP) hydrolysates and its fractions (Mean ± SE).

Fractions	CA	CC	CP
	ABTS (% Inhibition)		
F_0	79.69 ± 0.10^{Db}	91.28 ± 0.13^{Dc}	70.90 ± 0.14^{Da}
F_1	70.57 ± 0.30^{Ab}	81.09 ± 0.18^{Ac}	62.85 ± 0.30^{ABa}
F_2	73.14 ± 0.21^{Bb}	84.14 ± 0.15^{Bc}	65.05 ± 0.11^{Ba}
F_3	74.66 ± 0.16^{Cb}	87.38 ± 0.15^{Cc}	67.52 ± 0.23^{Ca}
F_4	72.43 ± 0.25^{ABb}	81.52 ± 0.21^{Ac}	62.24 ± 0.22^{Aa}
	DPPH (% Inhibition)		
F_0	32.25 ± 0.20^{Bb}	37.65 ± 0.15^{Cc}	28.38 ± 0.19^{Ba}
F_1	27.69 ± 0.19^{Ab}	33.66 ± 0.17^{Ac}	24.39 ± 0.18^{Aa}
F_2	27.92 ± 0.15^{Ab}	34.46 ± 0.23^{ABc}	24.92 ± 0.17^{Aa}
F_3	28.11 ± 0.30^{Ab}	35.09 ± 0.16^{ABc}	25.39 ± 0.12^{Aa}
F_4	29.88 ± 0.32^{ABb}	35.71 ± 0.17^{Bc}	27.38 ± 0.18^{Ba}
	FRAP (mM equivalent to $FeSO_4 \cdot 7H_2O$)		
F_0	20.38 ± 0.16^{Cb}	24.58 ± 0.13^{Dc}	18.83 ± 0.23^{Da}
F_1	16.09 ± 0.17^{Ab}	17.53 ± 0.11^{Bc}	11.68 ± 0.15^{Ba}
F_2	18.13 ± 0.09^{Bb}	20.19 ± 0.15^{Cc}	15.29 ± 0.14^{Ca}
F_3	19.65 ± 0.14^{Cb}	20.54 ± 0.11^{Cc}	15.53 ± 0.12^{Ca}
F_4	18.72 ± 0.09^{Bc}	15.34 ± 0.22^{Ab}	10.13 ± 0.18^{Aa}

Mean ± SE values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) do not differ significantly ($P < 0.05$) (n = 9).
CA: CCP hydrolyzed with Alcalase (6 h); CC: CCP hydrolyzed with α-Chymotrypsin (4 h); CP: CCP hydrolyzed with Papain (6 h); F0: whole hydrolysates; F1: fraction having peptide size (MW) in the range of <1 kDa MW; F2: fraction having peptide size (MW) in the range of 1–5 kDa; F3: fraction having peptide size (MW) in the range of 5–10 kDa and F4 fraction having peptide size (MW) in the range of >10 kDa.

the highest ABTS radical scavenging activity in the same group. This might be due to synergistic effect of both smaller as well as larger peptides, which were present in whole hydrolysate. Among different ultrafiltration products, the fraction F3 (5–10 kDa) had highest ABTS activity. These findings were in accordance with the findings of Salami et al. (2011). It suggested that the antioxidant activities of protein hydrolysate depend not only on their amino acid composition, but also on the size and sequence of their amino acids. This statement also supports the higher activity of the α-Chymotrypsin hydrolysate group (CC). This might also be due to the enzyme specificity to the particular site in the peptide chain. Studies have shown that free amino acids have reduced antioxidant activity than casein hydrolysates, which means that the primary structure of casein plays an important role (Silk et al., 1973; Imondi and Stradley, 1974).

DPPH has commonly been used in the analysis of antioxidant activity and the test system can be used for the primary characterization of the scavenging potential of peptides. The DPPH scavenging activity was assayed as an additional measure for assessing antioxidant activity of the whole hydrolysate as well as its fractions. The findings of DPPH assay of CCP hydrolysates with different enzymes are presented in Table 1. In the group CA, the DPPH activity of F0 (32.25 ± 0.20) was recorded significantly ($P < 0.05$) higher as compared to other groups, but was comparable to fraction F4 (29.88 ± 0.32). In the group CP, F0 and F4 were comparable but significantly ($P < 0.05$) higher than F1, F2 and F3 fractions. In CC group, DPPH was significantly ($P < 0.05$) higher in F0 than all other treatments. Among all the three groups i.e, CA, CC and CP, DPPH activity was recorded highest for CC, irrespective of the fractions. The variation in the DPPH inhibition might be due to the differences in the enzyme specificity leading to varying efficiency and cleavage site in proteins as well as presence or absence of functional groups such as hydroxyl groups on phenolic compounds (Cumby et al., 2008). Kamau and Lu (2011) also reported that the DPPH radical scavenging activity of the whey protein hydrolysate was dependent on the enzyme used as well as the hydrolysis conditions. Previous studies also demonstrated that a number of food-derived peptides or protein hydrolysates were capable of interacting and quenching DPPH radicals (Hogan et al., 2009; Kumar et al., 2016a).

Ferric reducing antioxidant power (FRAP) is a measurement of compound's ability to reduce ferric iron (III)/ferricyanide complex to ferrous iron (II). FRAP activity (mM equivalent to $FeSO_4 \cdot 7H_2O$) was also used to compare the antioxidant activity of the CCP hydrolysates and its fractions. In the group CA, the FRAP activity for the fractions F0 and F3 were comparable to F2 and F4, whereas F1 recorded lowest FRAP activity among all fractions. Among the fractions of group CC, the highest FRAP activity was recorded for F0 (24.58 ± 0.13) followed by F3, F2, F1 and F4. In the group CP, FRAP activity was comparable in F2 and F3 and the whole hydrolysate (F0) recorded highest FRAP activity (18.83 ± 0.23), whereas F4 lowest. The higher activity of the whole hydrolysates might be because of the higher concentration of peptide with varying molecular size and sequence as well as the structural functionality of constituent peptides which may have synergistic effects in scavenging the free radicals. Among all the enzymatic groups, the fractions derived out of α-Chymotrypsin hydrolysates (CC) had significantly ($P < 0.05$) higher FRAP activity. The difference in the free radical reducing activity for hydrolysates with different enzymes might be attributed to the enzyme specificity and the degree of hydrolysis of the constituent proteins, which ultimately produce hydrolysate with different amino acid composition. Klompong et al. (2007) also reported that the degree of hydrolysis and enzymes used for hydrolysis play vital role in the determination of reducing power of protein hydrolysates. Wu et al. (2003) reported that differences in antioxidant activity of protein hydrolysates might be attributed to the specific peptide/amino acid composition.

3.2. Antimicrobial activity of CCP hydrolysates and its fractions

The agar diffusion assay (or inhibition zone assay) is a common method used to test the antimicrobial activity of commonly used food antimicrobials/peptides/hydrolysates. The antibacterial activities of all enzymatic digested sample were evaluated against Gram-positive (*Listeria monocytogenes, Bacillus cereus, and Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. The efficacy of CCP hydrolysates/fractions for its antibacterial activity were measured in terms of zone of inhibition (mm) and values are depicted in Table 2.

Among the fractions of group CA, the highest antimicrobial activity (inhibition zone) against *S. aureus* was recorded for F0 (14.88 ± 0.41) followed by F4, F3, F2 and F1. In the CC group, the whole hydrolysate (F0) also exhibited significantly ($P < 0.05$) higher inhibitory effect against *S. aureus* than other fractions, however, the inhibitory effect were comparable for the remaining four fractions (13.68-15.63 mm). The fraction obtained from the group CP also exhibited lower antimicrobial activity than the whole hydrolysate (F0) (12.46 ± 1.09). The fraction F1 exhibited significantly ($P < 0.05$) lower antimicrobial activity as compared to the other fractions however, it was comparable to F2 and F3. In all the groups, F0 of CC exhibited significantly ($P < 0.05$) higher inhibition effect against *S. aureus* than that of CA and CP. However, the inhibitory activity of other fractions of CA and CC was comparable, whereas it was significantly ($P < 0.05$) lower for CP fractions.

Antimicrobial activity of CCP hydrolysates and its fractions were also measured against *E. coli,* the most common contaminating microorganism in food chain. Casein hydrolysate produced by Alcalase (CA) showed highest inhibitory activity (17.93 ± 0.82) against *E. coli* and was comparable to CC (17.62 ± 0.73) but CP showed significantly ($P < 0.05$) lower inhibitory activity. Among the fractions of CA, F4 had highest (16.37 ± 0.56) inhibitory activity followed by F3, F2 and F1. On comparison of fractions of CC and CP, a similar trend was observed in all the fractions, however, fractions derived from CC had higher antimicrobial effect against *E. coli*.

L. monocytogenes, one of the most important pathogenic microorganism associated with contamination of raw and pro-

Table 2
Antimicrobial activity (zone of inhibition in mm) of camel casein protein (CCP) hydrolysates and its fractions (Mean ± SE).

Fractions	CA	CC	CP
	S. aureus		
F_0	14.88 ± 0.41^{Ca}	19.11 ± 0.91^{Bb}	12.46 ± 1.09^{Ca}
F_1	12.46 ± 0.24^{Ab}	13.68 ± 0.90^{Ab}	8.45 ± 0.74^{Aa}
F_2	13.03 ± 0.30^{Ab}	14.45 ± 0.89^{Ab}	9.67 ± 0.74^{ABa}
F_3	13.43 ± 0.30^{ABb}	14.93 ± 0.88^{Ab}	10.43 ± 0.70^{ABa}
F_4	14.55 ± 0.58^{BCb}	15.63 ± 0.75^{Ab}	11.07 ± 0.82^{BCa}
	E. coli		
F_0	17.93 ± 0.82^{Cb}	17.62 ± 0.73^{Cb}	14.20 ± 0.40^{Ca}
F_1	14.18 ± 0.43^{Ab}	12.65 ± 0.60^{Ab}	10.20 ± 0.40^{Aa}
F_2	14.92 ± 0.49^{ABb}	14.40 ± 0.78^{ABb}	11.50 ± 0.44^{ABa}
F_3	15.30 ± 0.80^{ABb}	16.00 ± 0.55^{BCb}	12.17 ± 0.69^{ABa}
F_4	16.37 ± 0.56^{BCb}	15.80 ± 0.45^{BCb}	13.05 ± 0.70^{BCa}
	L. monocytogens		
F_0	15.52 ± 0.78^{B}	16.32 ± 0.77	15.12 ± 0.56^{C}
F_1	10.84 ± 0.82^{Aa}	14.27 ± 0.60^{b}	8.67 ± 0.82^{Aa}
F_2	13.38 ± 0.61^{Bab}	14.45 ± 0.64^{b}	12.10 ± 0.76^{Ba}
F_3	14.87 ± 0.62^{Bb}	15.75 ± 0.86^{b}	12.40 ± 0.80^{Ba}
F_4	15.10 ± 0.95^{Bb}	15.40 ± 0.72^{b}	13.47 ± 0.56^{BCa}
	B. cereus		
F_0	18.95 ± 0.34^{Dc}	17.65 ± 0.27^{Db}	13.13 ± 0.29^{Da}
F_1	13.43 ± 0.43^{Bc}	12.30 ± 0.17^{Ab}	8.65 ± 0.13^{Aa}
F_2	15.40 ± 0.29^{Cc}	13.37 ± 0.31^{Bb}	10.47 ± 0.38^{Ba}
F_3	13.02 ± 0.40^{ABb}	14.10 ± 0.46^{BCb}	10.70 ± 0.32^{Ba}
F_4	12.25 ± 0.27^{Aa}	14.85 ± 0.36^{Cb}	12.05 ± 0.34^{Ca}

Mean ± SE values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) do not differ significantly ($P < 0.05$) ($n = 9$).
CA: CCP hydrolyzed with Alcalase (6 h); CC: CCP hydrolyzed with α-Chymotrypsin (4 h); CP: CCP hydrolyzed with Papain (6 h); F0: whole hydrolysates; F1: fraction having peptide size (MW) in the range of <1 kDa MW; F2: fraction having peptide size (MW) in the range of 1–5 kDa; F3: fraction having peptide size (MW) in the range of 5–10 kDa and F4 fraction having peptide size (MW) in the range of >10 kDa.

cessed meat products, was also included in this study. The anti-listeria activity of whole hydrolysate and its fractions of CC did not differ significantly and the inhibition zone ranged from 14.27 ± 0.60 for F1 to 16.32 ± 0.77 mm for F0. In the group CA, fraction F1 exhibited significantly ($P < 0.05$) lower activity than other fractions where the inhibition effect were comparable. Among the fractions of group CP, the inhibition effect were comparable for F2, F3 and F4 ranging from 12.10–13.47 mm, whereas F0 recorded highest (15.12 ± 0.56) and F1 lowest (8.67 ± 0.82) among fractions against *L. monocytogenes*.

B. cereus is another important bacterium related to food contamination. Therefore, this organism was also included in our study to conduct antimicrobial assay of CCP hydrolysates and its fractions. Among the fractions of group CA, the inhibition zone of F3 (13.02 ± 0.40) was comparable to that of F1 and F4, however, these values were significantly ($P < 0.05$) lower than F2 and F0. The inhibition effects of F0 and F2 were also significantly different. Among the fractions of group CC, the zone of inhibition were comparable for F2, F3 and F4 ranging from 12.37–14.85 mm and the fraction F0 had significantly ($P < 0.05$) higher (17.65 ± 0.27) and F1 had significantly lower (12.30 ± 0.17) inhibition effects against *B. cereus*. A similar trend was also observed among the fractions of group CP, where the inhibition effects were comparable for F2 and F3 whereas the other fractions had significantly different inhibition effect. Among the fractions of respective group, the whole hydrolysate (F0) exhibited higher inhibition effect against *B. cereus*.

In this study, it was observed that the whole hydrolysates of each group exhibited significantly higher antimicrobial activity against the entire microorganism tested than its fractions. These differences in antimicrobial activity of whole hydrolysates and its fractions and also with different enzymes might be due to the differences in the size, ionic nature and concentration of the peptides present in the solution because it has been reported that the antibacterial efficacy of the bioactive peptides depend on several factors, including the load, structural diversity (Gennaro and Zanetti, 2000), hydrophobicity (Kustanovich et al., 2002), and specific amino acid composition, such as histidine, arginine, proline, cysteines and glycine (Andreu and Rivas, 1998). The higher antimicrobial activity of whole hydrolysates might also be due to presence of different peptides of various sizes and charges which might contributed synergistically to its activity. These results were in accordance with the findings of Gobbetti et al. (2004) who documented that the total antibacterial effect of milk was higher than the sum of individual contributions made by protein defences, which could be attributed to the synergistic activity between natural proteins and peptides and peptides from precursors. Benkerroum et al. (2004) also reported antimicrobial activity of camel's milk against pathogenic strains of *E. coli*, *L. monocytogenes and B. cereus* The results of the well diffusion assay *L. monocytogenes* LMG 13304 and *E. coli* O78:K80 (JB2) were the most sensitive as judged by the diameters of the inhibition zones. Recio and Visser (1999) reported that pepsin digestion of bovine αs_2-casein released two antibacterial domains, f(164–179) and f(183–207), which were strongly inhibitory to *E. coli*, *Bacillus cereus* and *Streptococcus thermophilus*.

Camels are famous for their ability to resist extreme stressful conditions, which presumably include their ability to resist infection in the mammary gland and digestive tract. Casein may serve as a source of peptides, which might support the innate immune system as already found in domestic ruminants (Silanikove et al., 2006; Leitner et al., 2006, 2011). Thus, in future studies, it would be interested to test the biological activity of peptides released from casein by the activity plasmin (the main proteolytic enzyme in milk) and trypsin (the first enzyme which attacks casein in the intestine). Both plasmin and trypsin are serine proteases. Interestingly, the most effective enzyme in terms of liberating peptides with biological activity was Alcalase, which is a serine protease, which support the above-described concept.

4. Conclusion

From this study, it can be concluded that camel milk casein hydrolysates could be fractionated using ultrafiltration technique to obtain peptides of different molecular weight range which could be utilized for assessing its activities and thereby its food application. However, in this study it was observed that the whole hydrolysates exhibited more functionality (both antioxidant and antimicrobial) as compared to fractions. This may be either synergistic effects of peptides of different size and molecular weight or the higher concentration of peptides in whole hydrolysates as compared to fractions. Proteases such as Alcalase, α-Chymotrypsin produced peptides with higher biological activity as compared to Papain. Results suggested that camel milk casein could be used as natural source of food protein to produce hydrolysates with higher antioxidant and antimicrobial activities. It also encourages the use of camel milk caseins and derived peptides for direct human consumption and as ingredient in nutraceutical and pharmaceuticals for enhancing its functionalities and shelf life.

Conflict of interest

There is no conflict of interest related to this manuscript.

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References

Agrawal, R.P., Swami, S.C., Beniwal, R., Kochar, D.K., Sahani, M.S., Tuteja, F.C., Ghouri, S.K., 2003. Effect of camel milk on glycemic control, risk factors and diabetes quality of life in type-1 diabetes: a randomised prospective controlled study. J. Camel Pract. Res. 10, 45–50.

Andreu, D., Rivas, L., 1998. Animal antimicrobial peptides: an overview. Biopolymers 47, 415–433.

Barzyka, W., Campagna, S., Wieclawc, K., Korchowiecc, B., Rogalskad, E., 2009. The affinity of two antimicrobial peptides derived from bovine milk proteins for model lipid membranes. Colloids Surfaces A 343, 104–110.

Benkerroum, N., Mekkaoui, M., Bennani, N., Kamal, H., 2004. Antimicrobial activity of camel's milk against pathogenic strains of *Escherichia coli* and *Listeria monocytogene*. Int. J. Dairy Technol. 57, 39–43.

Benzie, I.F.F., Strain, J.J., 1999. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Method Enzymol. 299, 15–27.

Brand-Williams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT- Food Sci. Technol. 28, 25–30.

Cumby, N., Zhong, Y., Naczk, M., Shahidi, F., 2008. Antioxidant activity and water-holding capacity of canola protein hydrolysates. Food Chem. 109, 144–148.

Deegan, L.H., Cotter, P.D., Hill, C., Ross, P., 2006. Bacteriocins: biological tools for bio-preservation and shelf-life extension. Int. Dairy J. 16, 1058–1071.

Gennaro, R., Zanetti, M., 2000. Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Biopolymers 55, 31–49.

Gobbetti, M., Minervini, F., Rizzello, C.G., 2004. Angiotensin I-converting enzyme-inhibitory and antimicrobial bioactive peptides. Int. J. Dairy Technol. 57, 173–188.

Hangerman, A.E., Riedl, K.M., Jones, G.A., Sovik, K.N., Ritchard, N.T., Hartzfeld, P.W., Riechel, T.L., 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agri. Food Chem. 46, 1887–1892.

Haque, E., Chand, R., Kapila, S., 2009. Biofunctional properties of bioactive peptides of milk origin. Food Rev. Int. 25, 28–43.

Hogan, S., Zhang, L., Li, J., Wang, H., Zhou, K., 2009. Development of antioxidant rich peptides from milk protein by microbial proteases and analysis of their effects on lipid peroxidation in cooked beef. Food Chem. 117, 438–443.

Hwang, J., Shyu, Y., Wang, Y., Hsu, C., 2010. Antioxidative properties of protein hydrolysate from defatted peanut kernels treated with esterase. Food Sci. Technol. 43, 285–290.

Imondi, A.R., Stradley, R.P., 1974. Utilization of enzymatically hydrolyzed soybean protein and crystalline amino acid diets by rats with exocrine pancreatic insufficiency. J. Nutr. 104, 793–801.

Kamau, S.M., Lu, R.R., 2011. The effect of enzymes and hydrolysis conditions on degree of hydrolysis and DPPH radical scavenging activity of whey protein hydrolysates. Curr. Res. Dairy Sci. 3, 25–35.

Kappeler, S.R., Farah, Z., Puhan, Z., 2003. 5′-Flanking regions of camel milk genes are highly similar to homologue regions of other species and can be divided into two distinct groups. J. Dairy Sci. 86, 498–508.

Klompong, V., Benjakul, S., Kantachote, D., Shahidi, F., 2007. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102, 1317–1327.

Korhonen, H., Pihlanto, A., 2006. Bioactive peptides: production and functionality—review. Int. Dairy J. 16, 945–960.

Kumar, D., Chatli, M.K., Singh, R., Mehta, N., Kumar, P., 2016a. Enzymatic hydrolysis of camel milk casein and its antioxidant properties. Dairy Sci. Technol. 96, 391–404.

Kumar, D., Verma, A.K., Chatli, M.K., Singh, R., Kumar, P., Mehta, N., Malav, O.P., 2016b. Camel milk: alternative milk for human consumption and its health benefits. Nutr. Food Sci. 46 (2), 217–227.

Kustanovich, D.E., Shalev, M., Mikhlin, L., Gaidukov Mor, A., 2002. Structural requirements for potent versus selective cytotoxicity for antimicrobial dermaseptin S4 derivatives. J. Biol. Chem. 277, 16941–16951.

Leitner, G., Krifucks, O., Merin, U., Lavi, Y., Silanikove, N., 2006. Interactions between bacteria type, proteolysis of casein and physico-chemical properties of bovine milk. Int. Dairy J. 16, 648–654.

Leitner, G., Merin, U., Silanikove, N., 2011. Effects of glandular bacterial infection and stage of lactation on milk clotting parameters: comparison among cows, goats and sheep. Int. Dairy J. 21, 279–285.

Lopez-Exposito, I., Amigo, A.L., Recio, I., 2007. Casein hydrolysates as a source of antimicrobial, antioxidant and antihypertensive peptides. Lait 87, 241–249.

Magjeed, N.A., 2005. Corrective effect of camel milk on some cancer biomarkers in blood of rats intoxicated with aflatoxin B_1. J. Saudi Chem. Soc. 9, 253–263.

Mao, X.Y., Cheng, X., Wang, X., Wu, S.J., 2011. Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis. Food Chem. 126, 484–490.

Marcuse, R., 1960. Antioxidant effect of amino acids. Nature 186, 886–887.

Meisel, H., 2005. Biochemical properties of peptides encrypted in bovine milk proteins. Curr. Med. Chem. 12, 1905–1919.

Recio, I., Visser, S., 1999. Identification of two distinct antibacterial domains within the sequence of bovine αs_2-casein. Biochim. Biophys. Acta 1428, 314–326.

Sakanaka, S., Tachibana, Y., 2006. Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effect on lipid oxidation in beef and tuna homogenates. Food Chem. 95, 243–249.

Salami, M., Moosavi-Movahedi, A.A., Moosavi-Movahedi, F., Ehsani, M.R., Yousefi, R., Farhadi, M., Niasari-Naslaji, A., Saboury, A.A., Chobert, J.M., Haertle, T., 2011. Biological activity of camel milk casein following enzymatic digestion. J. Dairy Res. 78, 471–478.

Silanikove, N., Merin, U., Leitner, G., 2006. Physiological role of indigenous milk enzymes: an overview of an evolving picture. Int. Dairy J. 16, 533–545.

Silk, D.B.A., Marrs, T.C., Addison, J.M., 1973. Absorption of amino acids from an amino acid mixture simulating casein and a tryptic hydrolysate of casein in man. Clin. Sci. 45, 715–719.

Suetsuna, K., Ukeda, H., Ochi, H., 2000. Isolation and characterization of free radical scavenging activities peptides from casein. J. Nutr. Biochem. 11, 128–131.

Umuhumuza, L.C., Wei-min, N., Sun, X., 2011. Effect of bovine lactoferrin and casein peptide powder on microbial growth and glucose utilization by microorganisms in pork meat during refrigerated storage at 4 °C. Pak. J. Nutr. 10, 208–213.

Wang, J., Wang, Y., Dang, X., Zheng, X., Zhang, W., 2013. Housefly larvae hydrolysate: orthogonal optimization of hydrolysis, antioxidant activity, amino acid composition and functional properties. BMC Res. Notes 6, 197–207.

Wu, H.C., Chen, H.M., Shiau, C.Y., 2003. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res. Int. 36, 949–957.