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Antioxidant and antimicrobial activity of ultra-filtered fractions of camel milk protein hydrolysates under *in-vitro* condition

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ABSTRACT

Sequential ultra-filtration technique was used to fractionate camel milk protein hydrolysates products by 3 different proteolytic enzymes, viz. alcalase, α-chymotrypsin and papain. The protein fractions were assessed 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 (inhibition zone assay). The whole hydrolysates recorded significantly higher inhibition activity in ABTS, DPPH and FRAP assay, whereas among fractions, F2 (1–5 kDa) and F3 (5–10 kDa) of all the 3 hydrolysates had higher activity. Higher antioxidant activities was also observed in α- chymotrypsin hydrolysates samples and its fractions followed by alcalase and papain. The zone of inhibition (mm) was also recorded higher for whole hydrolysates as compared to their fractions; however, different fractions had almost comparable antimicrobial effect. The protein hydrolyastes with alcalase and α- chymotrypsin recorded comperatively higher antimicrobial activity. The findings suggested that camel milk proteins could be valuable source to produce protein hydrolysates and ultra-filtration technique could also be used to get specific molecular weight peptides, however, for application in processed food or for direct human consumption, use of whole hydrolysates could be more beneficial and cost effective.

Key words: Antimicrobial activity, Antioxidant activity, Camel milk protein, Enzymatic hydrolysis, Ultra-filtration

Milk proteins are one of the readily available sources of essential amino acids required for growth and maintenance of newborn. It also plays an important role in protection from various disease and thereby promotion of human health (Meisel 2005). Arrays of encrypted peptides are present in intact milk proteins that can be released by fermentation and enzymatic hydrolysis. Recently, functionalities of some of the protein hydrolysates and peptides have also been explored as food additives in formulation of nutraceutical and pharmaceutical products.

The health promoting effects of food derived bioactive peptides on human health has been documented by many researchers since few decades, however, the antioxidant activity of peptides derived from dietary proteins was reported for the first time by Marcuse (1960). Thereafter, various protein sources have been explored for its antioxidant properties viz. milk casein (Suetsuna *et al.* 2000, Kumar *et al.* 2016a, Kumar *et al.* 2016c), 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).

A broad range of antimicrobial activity of bioactive peptide food origin has been reported against common microorganisms related to food spoilage and/or health significance. As food derived peptides are produced from harmless and inexpensive sources, it offers a great advantage over peptides derived from other sources for application in food products (Kumar *et al.* 2016c, 2017). Hence, there is a growing interest in the utilization of these bioactive peptides as food grade bio-preservatives or as health-promoting food supplements in the food processing industry. Antimicrobial peptides (AMP) mostly act as bactericidal. All AMPs act on cell wall or membrane of bacteria. AMPs have an affinity to the anionic phospholipids and lipopolysaccharides present in cell walls and membranes of bacteria (Barzyka *et al.* 2009).

The differences in functional and biological properties of dromedary camel (*Camelus dromedaries*) milk from other milk might be due to its specific chemical composition and structure of its protein components (Kumar *et al.* 2016b). Significant therapeutic attributes of camel milk in

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human disease conditions have been suggested by many researchers (Agrawal *et al.* 2003, Magjeed 2005, Mal *et al.* 2006), 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 proteins 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 (2016). Commercial production of bioactive peptides from milk proteins has been limited by a lack of suitable large-scale technologies. However, membrane separation technique is utilized to separate the peptides with a specific molecular weight range. Step-wise 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 ultra-filtration and to investigate its antioxidant and antimicrobial properties.

MATERIALS AND METHODS

Chemical and reagents: Fine chemicals such as 2,2–azinobis(3–ethylbenzthiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma–Aldrich Chemical Co. India. 2,4,6-tripyridyl-s-triazine (TPTZ) was purchased from MP Biomedicals, India. The dehydrated microbiological media 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, India.

Fractionation of hydrolysed camel protein solutions: The spray dried skim milk powder was reconstituted and hydrolysis experiment was carried out using different enzymes as reported by Kumar *et al.* (2016*a*). The protein hydrolystes (with alcalase: SA, α-chymotrypsin: SC and papain: SP) were sequentially ultra-filtered 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. 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, viz. 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 –20ºC till further analysis.

Antioxidant activity assay: The $ABTS^+$ radical scavenging activity was determined according to method described by Kumar *et al.* (2016a). The DPPH radical scavenging activity was estimated following the method of Brand-Williams *et al*. (1995) with slight modification. Briefly, 1 ml of DPPH reagent (100 μM) was mixed with 0.25 ml of 0.1M 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 H1 Hybrid 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) = $100–[(At_{20}/At_0) \times 100]$. The FRAP was assessed according to Benzie and Strain (1999) using multimode reader. Ferrous sulphate was used as standard for standard curve preparation.

Anti-microbial 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) was uniformly distributed by pouring 15–20 ml of pre-sterilized media and allowed for solidification. In each plate, 3 wells (8–10 mm diameter) were made using sterile cork borer. About 100 μl of each hydrolysates and fractions (protein concentration: 25–30 mg/ml) were poured into well onto solid media in nutrient agar for all the test organisms and incubated at 37ºC for 24 h. The diameters of inhibitory zone surrounding the wells were measured using digital vernier calipers.

Statistical analysis: The experiments were conducted for 3 times and recordings were analysed in triplicate (9). Data were expressed as means with standard error. 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).

RESULTS AND DISCUSSION

The protein hydrolysates with alcalase (SA), α-chymotrypsin (SC) and papain (SP) were ultra filtered to get the different fractions, viz. F0: whole hydrolysates, F1: <1 kDa, F2: 1–5 kDa, F3: 5–10 kDa and F4: >10 kDa.

Antioxidant activity of camel milk protein hydrolysates

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

Fraction	SA	SC	SP
	ABTS (% Inhibition)		
F0	76.88±0.16^{Cb}	89.02±0.11^{Dc}	72.90±0.27^{Ca}
F1	67.62±0.22^{Ab}	83.5±0.13^{Ac}	61.83±0.28^{Aa}
F2	71.76±0.18^{Bb}	85.05±0.16^{Bc}	65.97±0.42^{Aba}
F3	73.05±0.16^{Bb}	86.85±0.12^{Cc}	67.24±0.21^{Ba}
F4	72.28±0.15^{Bb}	83.90±0.17^{Ac}	65.21±0.22^{Aba}
	DPPH (% Inhibition)		
F0	29.30±0.16^{Ba}	39.66±0.12^{Db}	30.49±0.14^{Cab}
F1	25.13±0.14^{Aa}	35.08±0.16^{Ab}	26.43±0.23^{Aab}
F2	25.78±0.18^{Aa}	36.05±0.13^{ABb}	27.01±0.18^{ABab}
F3	25.81±0.09^{Aa}	36.43±0.18^{Bc}	28.15±0.20^{ABb}
F4	28.63±0.12^{Ba}	37.69±0.17^{Cb}	28.83±0.21^{BCa}
	FRAP (mM equivalent to $FeSO_4.7H_2O$)		
F0	20.67±0.10^{Da}	24.31±0.05^{Db}	20.20±0.13^{Ea}
F1	14.32±0.10^{Ba}	16.01±0.11^{ABb}	15.83±0.11^{Bb}
F2	15.24±0.11^{Ca}	16.31±0.13^{Bab}	17.13±0.09^{Cb}
F3	15.46±0.09^{Ca}	17.20±0.10^{Cb}	17.95±0.11^{Db}
F4	9.82±0.10^{Aa}	15.58±0.10^{Ac}	12.81±0.13^{Ab}

Mean±SE values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) do not differ significantly (P<0.05). SA, Camel milk protein hydrolyzed with Alcalase (6 h); SC, Camel milk protein hydrolyzed with α-Chymotrypsin (4 h); SP, Camel milk protein hydrolyzed with Papain (6 h); F0, whole hydrolysates; F1, fraction having peptide size (MW) <1 kDa; 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) >10 kDa.

and its fractions: The group SC had significantly (P<0.05) higher ABTS activity than other 2 groups (Table 1). Among the fractions of group SA, the ABTS activity of the whole hydrolysate (F0) was significantly (P<0.05) higher than all other fractions. In group SC, the ABTS activity significantly (P<0.05) varied among fractions and highest activity was recorded for F0 followed by F3, F2, F4 and F1. The ABTS activity of fraction F0 of group SP was also significantly (P<0.05) higher as compared to other fractions, however, the fractions F2, F3 and F4 exhibited comparable activity. The ABTS radical scavenging activity of whole hydrolysate was recorded highest, irrespective of enzyme used. This might be due to the presence of wide range of peptides of different molecular weight as well as the higher concentration of peptides and free amino acids in whole hydrolysates. The fraction F3 (5–10 kDa MW) exhibited higher activity as compared to other fractions of same group. This might be due to greater charge and presence of more reactive groups on unfolded peptide chain.

The DPPH activity of F0 and F4 of SA were comparable, but were significantly (P<0.05) higher than the other fractions. Among fractions and whole hydrolyastes of the group SC, the DPPH activity for F1, F2 and F3 were comparable and the activity for F4 was recorded significantly (P<0.05) higher than other 3 fractions. The whole hydrolysate (F0) recorded highest DPPH activity. In the group SP, the DPPH inhibition activity of F1 was found to be lowest among all fractions, but was comparable to the activity of F2 and F3. Among all the three groups, i.e. SA, SC and SP, DPPH activity was recorded significantly (P<0.05) higher for SC and for all the fractions. The variation in the DPPH inhibition might be due to the use of different enzymes for protein hydrolysis, which might affect antioxidant potential of resultant hydrolysates (Kumar *et al.* 2016c) as enzyme specifity has particular cleavage site leading to the production of peptides with 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.

On comparison of the FRAP assay of fractions of camel milk hydrolysates, FRAP values were measured highest for F0 and lowest for F4, irrespective of type of enzyme and molecular weights of peptide fractions. Among the factions of group SA, the FRAP activity of F2 and F3 were comparable but significantly lower than that of F0 and significantly higher than F1 and F4. In the group SC, the FRAP values for the fractions F1, F2 and F4 were comparable, but were significantly (P<0.05) lower than that of F0 and F3. Different fractions of group SP ranged between 12.81±0.13 (F4) to 20.20±0.13 (F0) mM equivalent to $FeSO_4.7H_2O$. The higher concentration of peptide and the synergistic effects in scavenging the free radicals as well as the structural functionality of constituent peptides might be the cause for higher activity of the whole hydrolysates (Kumar *et al.* 2016c). 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 produces hydrolysate with different amino acid composition.

Antimicrobial activity of camel milk protein hydrolysates and its fractions: The camel milk protein hydrolysate produced by alcalase (SC) showed highest inhibitory activity against *S. aureus* among all the 3 groups (Table 2). Among the fractions of SA, F0 had highest inhibitory activity followed by F4, F2, F3 and F1. When comparing the inhibitory effect of fractions of SC, the fractions F1, F2 and F3 had comparable inhibition activity whereas F0 and F4 had significantly higher activity. In the group SP, a similar trend was also observed in all the fractions. Several authors also reported antimicrobial effect of milk derived peptides (McCann *et al.* 2005, Hayes *et al.* 2006, Kumar *et al.* 2016d).

Antimicrobial assay of camel milk protein hydrolysates and its fractions was also conducted against *E. coli* and whole hydrolysates (F0) of all the group exhibited significantly (P<0.05) higher inhibition effect as compared to its other fractions.

In general, the anti-listerial activity of F0 protein hydrolysate fractions was highest irrespective of type of

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

Fraction	SA	SC	SP
	S. aureus		
F0	15.58±0.70^{Ca}	18.88±1.06^{Cb}	13.96±0.88^{Ca}
F1	10.33±0.66^{Aa}	13.31±0.62^{Ab}	9.75±0.71^{Aa}
F2	13.6±1.07^{BC}	13.53±0.46^{A}	10.96±0.95^{AB}
F3	12.36±0.35^{ABab}	14.46±0.81^{ABb}	11.78±0.84^{ABa}
F4	15.00±0.90^{Cab}	16.20±0.59^{Bb}	12.81±0.86^{BCa}
	E. coli		
F0	15.42±0.72^{Ba}	17.80±0.75^{Cb}	13.40±0.53^{Ba}
F1	10.48±0.78^{A}	11.22±0.66^{A}	9.92±0.69^{A}
F2	13.40±0.75^{Bb}	14.62±0.46^{Bb}	10.82±0.99^{Aa}
F3	14.82±0.53^{Bb}	14.87±0.66^{Bb}	11.23±0.41^{Aa}
F4	14.93±0.61^{Bb}	15.60±0.40^{Bb}	11.57±0.75^{ABa}
	L. monocytogenes		
F0	16.30±0.67^{Bab}	17.45±0.73^{Cb}	14.85±0.82^{Ca}
F1	12.27±0.73^{A}	11.70±0.61^{A}	10.87±0.65^{AB}
F2	11.93±0.38^{Ab}	12.95±0.65^{Ab}	9.57±0.80^{Aa}
F3	11.85±0.72^{A}	13.48±0.82^{AB}	12.13±0.87^{B}
F4	12.45±0.57^{Aa}	15.37±0.44^{Bb}	11.80±0.63^{ABa}
	B. cereus		
F0	16.42±0.46^{Db}	15.67±0.26^{Db}	11.57±0.31^{Ca}
F1	11.87±0.30^{Bc}	10.90±0.32^{Ab}	8.62±0.18^{Aa}
F2	14.03±0.36^{Cc}	12.70±0.23^{Bb}	8.48±0.17^{Aa}
F3	12.32±0.45^{Bb}	14.42±0.30^{Cc}	10.17±0.29^{Ba}
F4	10.34±0.32^{Aa}	12.45±0.53^{Bb}	9.27±0.48^{Aa}

Mean±SE values bearing same superscripts row-wise (small alphabets) and column-wise (capital alphabets) do not differ significantly ($P<0.05$). SA, Camel milk protein hydrolyzed with Alcalase (6 h); SC, Camel milk protein hydrolyzed with α-Chymotrypsin (4 h); SP, Camel milk protein hydrolyzed with Papain (6 h); F0, whole hydrolysates; F1, fraction having peptide size (MW) <1 kDa; 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) >10 kDa.

enzyme and molecular weight. However, F0 of SC exhibited highest (17.45±0.73) listerial inhibition zone among all the groups. In SA group, all the fractions exhibited comparable activity. In SC group, F1 and SP group, F2 displayed lowest size inhibition zones.

On similar lines with other organisms, *B. Cereus* was also inhibited maximum by F0 fraction between the groups and SC fraction among the groups. However, critical appraisal of results indicated that papain could not produce good antimicrobial peptides on hydrolysis of camel milk proteins. We observed that all the fractions of each group exhibited significantly lower antimicrobial activity against the entire microorganism tested than whole hydrolysates. These differences in antimicrobial activity of fractions and whole hydrolysates 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 the antimicrobial efficacy of the bioactive peptides depend on several factors, including the load, structural diversity (Gennaro and Zanetti 2000), hydrophobicity (Kustanovich *et al.* 2002), and presence of specific amino acid, 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 have 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.

From this study, it can be concluded that fractionation of camel milk protein hydrolysates could be achieved by using ultrafiltration technique and could be utilized for assessing its activities and thereby its food application. However, the whole hydrolysates exhibited more functionality (both antioxidant and antimicrobial) as compared to its fractions. This might be either due to synergistic effects of peptides of varying size and molecular weight or the higher concentration of peptides in whole hydrolysates as compared to fractions. Higher biological activity of protein hydrolysates could be achieved by hydrolysis with proteases such as alcalase, α-chymotrypsin. Results suggested that camel milk proteins 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 and milk proteins, and derived peptides for direct human consumption and as ingredient in processed foods, nutraceutical and pharmaceuticals products.

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