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

Devendra Kumar[b,*], Manish Kumar Chatli[a], Raghvendar Singh[b], Nitin Mehta[a], Pavan Kumar[a]

[a] Department of Livestock Products Technology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141 004, Punjab, India
[b] ICAR—National Research Centre on Camel, P.B. No. 07, Bikaner, Rajasthan, India

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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

* Corresponding author.
E-mail address: drdev24@gmail.com (D. Kumar).

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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-