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Improvement of the Antimicrobial and Antioxidant Activities of Camel and Bovine Whey Proteins by Limited Proteolysis.

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The compositions and structures of bovine and camel milk proteins are different, which define their functional and biological properties. The aim of this study was to investigate the effects of enzymatic hydrolysis of camel and bovine whey proteins (WPs) on their antioxidant and antimicrobial properties. After enzymatic treatment, both the antioxidant and the antimicrobial activities of bovine and camel WPs were improved. The significantly higher antioxidant activity of camel WPs and their hydrolysates as compared with that of bovine WPs and their hydrolysates may result from the differences in amounts and/or in accessibilities of antioxidant amino acid residues present in their primary structures and from the prevalence of α-lactalbumin and β-lactoglobulin as proteolytic substrates in camel and bovine whey, respectively. The results of this study reveal differences in antimicrobial and antioxidant activities between WP hydrolysates of bovine and camel milk and the effects of limited proteolysis on these activities.

KEYWORDS: Camel whey proteins; antioxidant activity; antimicrobial properties; proteolysis

INTRODUCTION

The concepts in nutrition have changed considerably during the past decade. Growing interest in functional foods, which apart from nutritional values might be health-promoting and reduce the risk of several of diseases, (*1*, *2*) is one of the stimuli for these changes. Whey proteins (WPs) are highly functional foods showing high protein quality scores and containing relatively high proportions of essential amino acids. Consequently, increasing attention has been focused on the production of bioactive peptides derived from WPs (*3*,*4*). Enzymatic hydrolysis of WPs may affect human health by improving some of their biological properties, including mineral binding, growth factors, reduction of blood pressure, antioxidant activity, anticancer activity, immunomodulatory function, opioid activity, cholesterol-lowering effects, and protective properties against different microorganisms and viruses (*5*−*8*). Much attention has been paid to the antioxidant activity of WPs. Although the exact antioxidant mechanism of WPs is still unknown, the elevated amount of sulfur-containing amino acids in WPs may be at the base of it. Moreover, antioxidant enzymes (e.g., lactoperoxydase, catalase) and lactoferrin (LF) participate actively in the enhancement of antioxidant capacity of WPs (*9*). The antioxidant activities of WPs and of their hydrolysates offer the potential application of these proteins for the enhancement of stability of food products by preventing their oxidative deterioration (*10*). Since natural antioxidants are readily accepted by consumers and are generally recognized as safe (GRAS), WPs and their hydrolysates have attracted particular interest (*11*). WPs from different species also contain various amounts of antimicrobial components. Many components found in WPs such as immunoglobulins (Igs), LF, lactoperoxydase, lysozyme, and *N*-acetyl-β-D-glucosaminidase exhibit antimicrobial activities (*3*). As the result of structural variability, the bioactive properties of WPs from the milk of different animal species may vary. Camel milk differs from bovine milk in both composition and structure of its protein components, which influences their functional and biological properties (*12*). The antimicrobial activity of camel's milk has already been studied (*13*, *14*), but to our knowledge, no study has been carried out on the antimicrobial and antioxidant activities of camel WP hydrolysates. Therefore, the present work was undertaken to study and compare the antimicrobial and antioxidant

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activities of WPs from camel and bovine sources after limited enzymatic proteolysis.

MATERIALS AND METHODS

Materials. Bovine milk was collected from the University farm, and camel milk was provided by the Department of Clinical Sciences and Faculty of Veterinary Medicine of the University of Tehran (Tehran, Iran). Chymotrypsin (EC 3.4.21.1; activity 45 unit mg^{-1} protein) from bovine pancreas, trypsin (EC 3.4.21.4; activity 13 500 unit mg^{-1} protein), thermolysin from *Bacillus thermoproteolyticus rokko* (EC 3.4.24.27; activity 50−100 units mg^{-1} protein), proteinase K from *Tritirachium album* (EC 3.4.21.64; activity 30 units mg^{-1} protein), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were obtained from Sigma−Aldrich Chemie GmbH (Munich, Germany). Other chemicals were of analytical grade (Sigma−Aldrich) and were used without further purification.

Preparation of WP/WP Hydrolysate Samples. Camel and bovine milk was warmed at 37 °C and skimmed immediately by centrifugation (5000*g*, 15 min). WPs were obtained after precipitation of caseins at pH 4.6 with 1 N HCl and centrifugation (5860*g*, 60 min, 4 °C). WPs were washed and centrifuged three times. The purity of WPs was checked using SDS-PAGE (data not shown). They were very pure and free from caseins. After the WPs were isolated from caseins and their pH was adjusted to 6.8 using 1 N NaOH, they were dialyzed against double-distilled water and then they were stored at −20 °C until use. Before each experiment WPs were dialyzed against 20 mM phosphate buffer at pH 7.8. For proteolysis experiments, both camel and bovine WP solutions were prepared in 20 mM phosphate buffer at pH 7.8. The incubation was carried out at 37 °C, at an enzyme/substrate ratio of 1/100 (w/w), up to 5 h. After each experiment, WPs and their hydrolysates were separated by using ultrafiltration (UF) membranes (Amicon Ultra-15, Millipore, cutoff of 10, 5, and 3 kDa) and centrifugation for 15 min at 1680*g* at 4 °C. Permeates of each stage of filtration were collected, lyophilized, and stored at −20 °C until further use.

Determination of Antioxidant Activities. The antioxidant activities of camel and bovine WPs and of their hydrolysates were measured according to the method described by Re et al. (*15*). To prepare the $ABTS^{\bullet+}$ radical, 7 mM ABTS solution was oxidized in water by treatment with 2.45 mM potassium persulfate (molar ratio of 1:0.5) for 12−16 h in the dark. The $ABTS^{\bullet+}$ solution was diluted in 5 mM phosphate buffer (pH 7.4) prior to assay, giving an absorbance of 0.70 ± 0.2 at 734 nm. A proper amount of sample was added to 1 mL of reagent and incubated at 25 °C. Scavenging of the $ABTS^{\bullet+}$ radical was monitored by an absorbance decrease at 734 nm using a spectrophotometer (Shimadzu, Model UV-3100, Kyoto, Japan). A reading was taken after 1 min of the initial mixing and up to 6 min periodically. A solvent blank was run in each assay. The water-soluble vitamin E analogue Trolox was used as standard. The results are mean values of triplicates.

Determination of Antimicrobial Activities. The assay was carried out in sterile honeycomb micro plates. In each well, 200 μL of overnight cultured *Escherichia coli* Dh1α and 50 μL of 0.5 mg mL^{-1} of WPs or their hydrolysates were added. As a control experiment, 200 μL of bacteria and 50 μL of 20 mM phosphate buffer were applied into the wells. The mixture was incubated at 37 °C for 16 h. The optical density at 600 nm was measured using an Elisa reader Expert 96 (ASYS Hitech, Eugendorf, Austria) every 1 h for the first 2 h and every 30 min until the end of the experiment. The specific growth rate was calculated from the plot of OD_{600} versus time ($\mu = \ln OD_{600}/\Delta t$), where t is time (*16*). The experiments were repeated five times for each sample.

Reversed-Phase High-Performance Liquid Chromatography Profiles. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed with an automated HPLC system (consisting of an analytical HPLC Knauer pump, a Model 2500 UV detector, monochromator spectrophotometer, wavelength 190−740 nm, an ODS-C_{18} column, 3−5 μm, 250 × 4.6 mm) to follow protease-catalyzed degradation of WPs. Conditions of elution were those used by Mota et al. (*17*). Gradient elution was carried out with a mixture of solvent A (0.1% trifluoroacetic acid (TFA) in water) and solvent B (0.1% TFA in 80% aqueous acetonitrile, v/v). Proteins and peptides were eluted as follows: 0−1 min, 90% A; 1−10 min, 90−80% A; 10−15 min, 80−75% A; 15−20 min, 75−60% A; 20−30 min, 60−50% A; 30−39 min, 50−20% A; 39−41 min, 20−0% A; return to initial conditions in 19 min. The flow rate was 0.5 mL min^{-1}. Elution was performed at room temperature (22 °C) and detection at 215 nm. Samples were filtered through 0.2 μM filters.

Protein and Peptide Assay. The protein concentration was determined by the Bradford method, with bovine serum albumin (BSA) as the protein standard (*18*). Each measurement was carried out three times, and the result is the average of three experiments. The peptide concentration in WP hydrolysates and UF fractions was determined by the OPA (*o*-phthaldialdehyde) method using tryptone as standard (*19*).

Statistical Analysis. In this study data are presented as mean value with standard deviations. The significance between mean values was determined statistically with the t test, p value < 0.05 using SPSS for Windows, version 16.

RESULTS AND DISCUSSION

Antioxidant Activities of WPs before and after Limited Proteolysis and Size-Based Fractionation. In this study the Trolox-equivalent antioxidant capacity (TEAC) values, based on the consumption of the colored ABTS radical, was used to calculate the antioxidant activity of WPs/WP hydrolysates. As shown in **Figure 1**, camel WPs/WP hydrolysates exhibit significantly higher antioxidant activities than bovine WPs/WP hydrolysates. Proteins owe their antioxidant activities to the presence of some amino acids in their primary structure (*20*). Many amino acid residues convey antioxidant activity to proteins either because of their capacities to donate protons to free radicals (Trp, Phe, Tyr, His, and Cys) or because of their capacities to chelate metal cations (Glu, Asp, Lys, Arg, and His) (*21*). Not only the content in antioxidant amino acids but also their correct positioning in the sequence are important factors determining the antioxidant properties (*20*). Thus, the significant differences in antioxidant activities of camel and bovine WPs/WP hydrolysates may result from different amounts and/or accessibility of antioxidant amino acid residues in their structures. It has been reported that the changes in functional properties of WPs are mainly characterized by a lower molecular weight, an exposure of hydrophobic groups, and an increased number of ionic groups (*22*). The aforementioned structural characteristics of WPs can be altered by limited proteolysis. As shown in **Figure 1**, the antioxidant capacities of both bovine and camel WPs were enhanced significantly after limited proteolysis. This finding can be explained by a better accessibility of the antioxidant residues and their easier access and better contribution in either redox reactions or metal chelating activities of lower molecular weight peptides after partial proteolysis of WP substrates. After a size-based fractionation, the highest antioxidant activities were obtained for permeate of 5 and 10 kDa UF membrane from camel WPs hydrolyzed with chymotrypsin, suggesting that antioxidant activities depend on the size and on the composition of peptides. Our results remain in agreement with those reported already (*23*), suggesting the dependence of antioxidant activities on the sizes of obtained peptides after the limited proteolysis of WPs. The peptide fractions of different sizes obtained after partial hydrolysis with trypsin and chymotrypsin of both bovine and camel WPs displayed the lowest and highest antioxidant activities, respectively. Chymotrypsin with specificity toward the carboxylic side of aromatic or other hydrophobic amino acid residues generates peptides with the antioxidant amino acids at their C-terminal position (*9*). In contrast, trypsin with specificity toward cleavage at basic amino acids Lys and Arg produces peptides with poorer antioxidant properties (*24*). Thus, these results suggest that C-terminal residues of antioxidant peptides play an important role in determining their antioxidant properties. Although all 20 amino acids have oxidizing value, the most reactive oxidable amino acids are Cys, Met, Trp, Tyr, and Phe. In the case of Cys

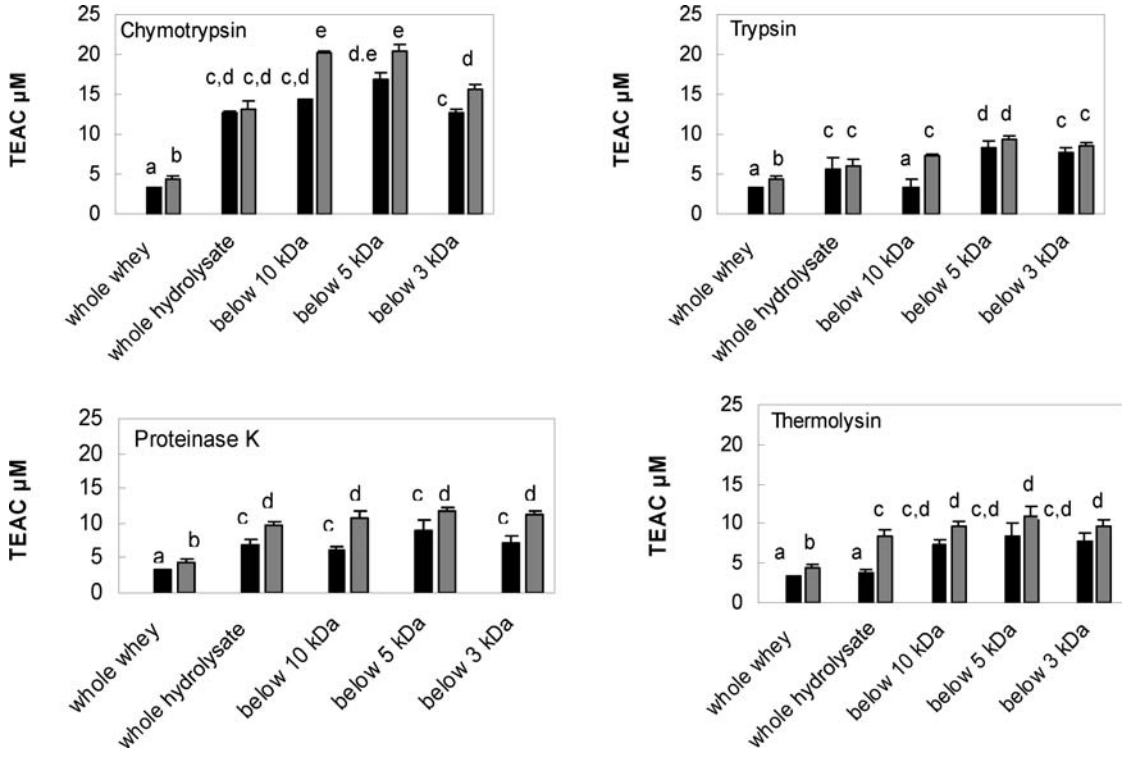

Figure 1. Measurement of size-dependent antioxidant activities of whey proteins/peptide fragments from bovine (black) and camel (gray) sources. Whole whey proteins (WPs) and peptide fractions obtained after enzymatic hydrolysis followed by size fractionation were used for measuring antioxidant activities. Proteinase K, thermolysin, trypsin, and chymotrypsin were used for the proteolysis of whey proteins. The data marked with different letters are significantly different ($P < 0.05$).

and Met, oxidation happens due to hydrogen abstraction from SH groups. For Trp, Tyr, and Phe, oxidation occurs by OH attack or one-electron oxidation of the aromatic ring (*25*). One of the whey proteins that has a high antioxidant activity is α-lactalbumin. α-Lactalbumin concentration in camel WPs is four times higher than in bovine WPs. Because camel α-lactalbumin has a higher amount of the aforementioned antioxidant amino acids compared to its bovine counterpart, it has a higher antioxidant activity (*26*). To our knowledge, there has been no report so far about the antioxidant activities of peptides obtained from camel whey proteins. The data presented here provide useful information about the possibilities of use of such antioxidant peptides in the production and development of functional food products.

Antimicrobial Properties of WPs before and after Limited Proteolysis and Size-Based Fractionation. There are an increasing amount of data mentioning in vitro antibacterial, antifungal, antiviral, and anticancer properties of milk-derived peptides (*3*). Both WPs and their hydrolysates were assessed for their antibacterial effects against *E. coli*. *E. coli* was cultured in Luria Broth medium with the addition of different WPs/WP hydrolysates. The specific growth rates were compared with the reference experiment (**Table 1**). Both bovine and camel WPs, and their hydrolysates, inhibit the growth of *E. coli*. Camel WPs revealed markedly greater antimicrobial activities than bovine WPs before hydrolysis. This finding can be explained by the higher content of antimicrobial factors such as lysozyme, lactoferrin, and immunoglobulins in camel milk (*14*, *27*). There is a great interest in camel milk immunoglobulins (IgGs), which are quite unique in the animal world and could be used to neutralize bacterial and viral enzymes (*27*). As shown in **Table 1**, the limited proteolysis performed with all proteolytic enzymes used in this study enhanced the antimicrobial activities of both camel and bovine

Table 1. Percentages of Growth-Specific Rate Reduction as Compared with Reference Experiment $((\mu_{ref} - \mu)/\mu_{ref}) \times 100$ [a]

		camel/bovine			
camel/bovine whole whey	enzyme	whole hydrolysate	10 kDa	5 kDa	3 kDa
16.4/4.5	proteinase K	47.3/18.2*	42.8/20.0*	44.5/27.3	49.1/30.9
16.4/4.5	thermolysin	26.4/7.3*	38.2/8.2*	27.2/8.3*	45.5/7.5*
16.4/4.5	chymotrypsin	20.0*/11.8*	20.9*/11.8*	20.0*/11.8*	21.0*/10.9*
16.4/4.5	trypsin	19.1/16.4	29.1/13.6*	27.3/13.6*	32.7/12.7

[a] Data marked with an asterisk are not significantly different ($P < 0.05$).

WPs. The highest inhibition of growth of *E. coli* was obtained by applying the hydrolysate of camel WP obtained after action of proteinase K and the UF permeate of 3 kDa. It is believed that the size of an active sequence is between 2 and 20 amino acid lengths; therefore, a peptide with a smaller size obtained from membrane fractionation should have a higher antimicrobial activity, since it might pass through the membrane of the bacteria more conveniently (*3*). The hydrolysates obtained after digestion of bovine and camel WPs with chymotrypsin and trypsin displayed similar patterns of antimicrobial activities. Thus, the obtained results can be explained also by the difference in both composition and structure between camel and bovine WPs.

RP-HPLC Profiles of WPs/WP Hydrolysates. In this study the peptide fractions obtained were used for further characterization using chromatography. RP-HPLC was used to follow the enzymatic degradation of camel and bovine WPs, at 37 °C, using trypsin, chymotrypsin, thermolysin, and proteinase K. One of the main differences between camel and bovine milk is that camel milk, as does human milk, lacks β-lactoglobulin (β-LG), the most allergenic protein found in bovine milk (*12*, *14*). Hence, its relative proportions are very different in these two wheys, with a clear

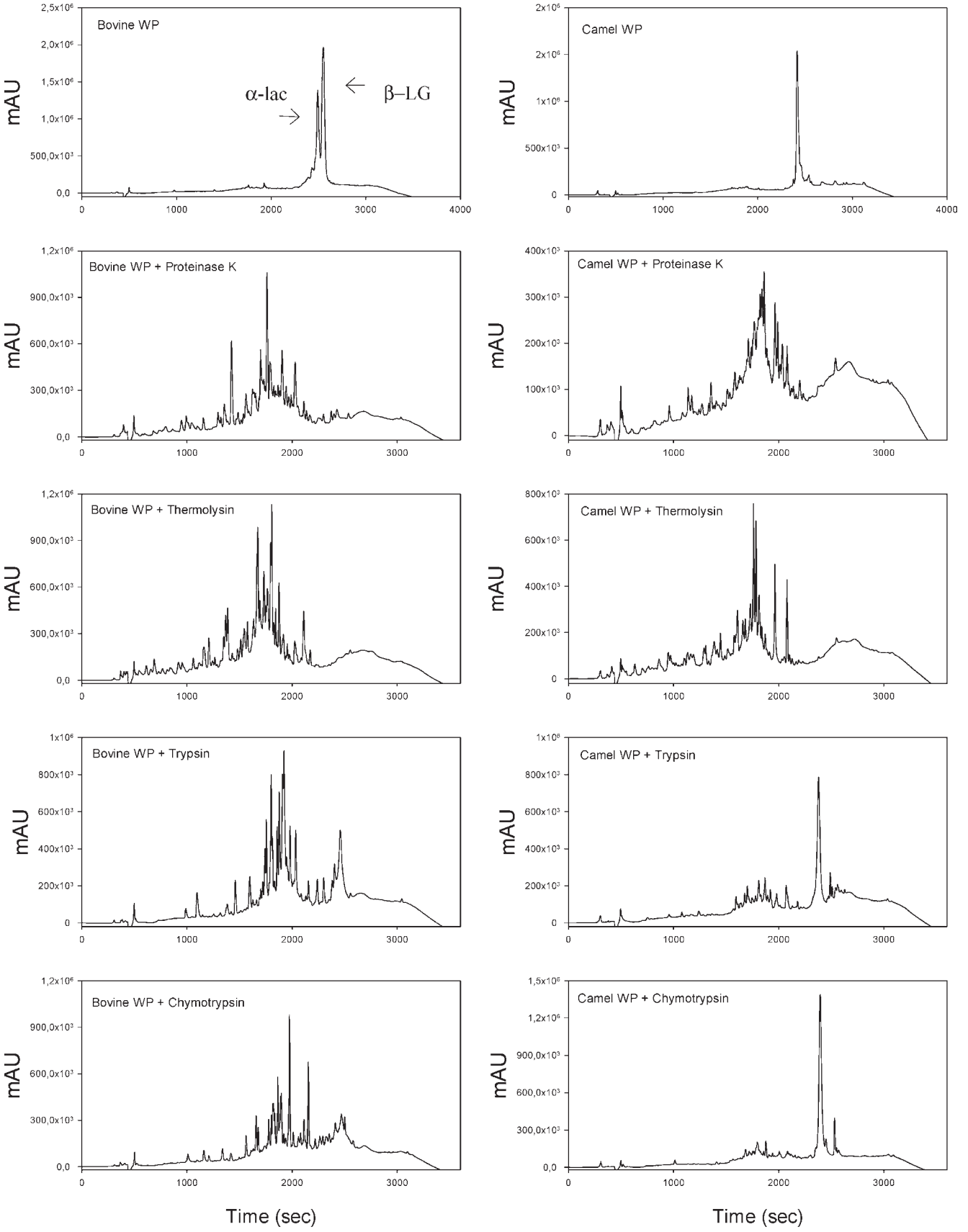

Figure 2. RP-HPLC profiles of whey protein/protein hydrolysates of bovine and camel milk obtained after hydrolysis by proteinase K, thermolysin, trypsin, and chymotrypsin at 37 °C for 5 h.

predominance of β-LG in bovine whey and dominance of α-lactalbumin (α-lac) in camel whey. Under the experimental conditions used in this study, α-lac and β-LG fractions were well separated with retention times of 41.6 and 42.5 min, respectively, in the case of bovine whey and 40 min in the case of camel α-lac. The appearance of peptides was followed as a function of time.

As shown in **Figure 2**, the resulting hydrolysates after the action of bacterial and digestive proteases were resolved into several major peaks presenting a wide range of polarities and sizes. Moreover, marked differences were observed in the hydrolysis patterns between bovine and camel WPs (**Figure 2**). Chromatograms of hydrolysates obtained after the action of microbial proteases displayed numbers of peptide fractions larger than those obtained after digestion with pancreatic enzymes, which can be explained by the broad specificity of microbial proteases toward these substrates. The main peaks corresponding to the major proteins in bovine and camel whey disappeared almost completely on treatment with bacterial enzymes, suggesting a more extensive hydrolysis of WPs with the prokaryotic proteases. As shown in **Figure 2**, the number of peaks obtained in the hydrolysates of camel whey was smaller than that obtained in the hydrolysates of bovine whey, especially after the action of pancreatic proteases, demonstrating the better resistance of camel WPs against used proteases. The differences in the proteolytic patterns between bovine and camel WPs come from differences in their structure and stability. In the hydrolysate of camel whey, the main protein fraction corresponding to α-lac displays notable resistance to digestive enzymes after 5 h of incubation, while this whey protein was extensively hydrolyzed by the bacterial proteases (**Figure 2**) under the same experimental conditions. The significantly higher stability of camel whey proteins as compared with that of bovine whey proteins was also observed in our previous study (*28*). As described above, the four proteases used for hydrolysis of bovine and camel WPs led to different patterns of degradation. Comparing the final patterns of hydrolysis, one can conclude that camel and bovine WPs have different peptide profiles mainly due to the lack of β-LG in camel whey. This may offer camel WPs as a novel candidate for further study with the possible application to functional foods.

In conclusion of this study focused on using the enzymatic hydrolysis of WPs to improve their antioxidant and antibacterial activities, it can be said that the obtained results demonstrate that such activities and composition of hydrolysate peptide fractions depend on the WP source and the enzymes used. The antioxidant and antibacterial activities of fractions obtained after ultrafiltration were different, suggesting that both the size and the composition of peptide fractions are important for specific activity. The hydrolysates obtained with camel WPs, lacking BLG and rich in lysozyme and α-lactalbumin, displayed antioxidant and antimicrobial activities significantly higher than those of hydrolysates of bovine WP, in which BLG is a predominant substrate. Our work therefore has shown that the enzymatic hydrolysis of WPs improves the biological properties and offers an interesting opportunity for food application. Despite the need for further research, camel whey proteins and their hydrolysates, especially those obtained by treatment with chymotrypsin, could be considered as suitable natural antioxidants for the prevention of oxidative reactions in the food industry and could become ingredients of functional foods.

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