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Identification of bioactive peptides derived from caseins, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), and peptidoglycan recognition protein-1 (PGRP-1) in fermented camel milk

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Abstract

Camel milk was fermented by *Streptococcus thermophilus* LMD-9 strain, the proteolytic system of which yielded peptides from the milk proteins. The peptides were isolated by cation-exchange chromatography and ultrafiltration, and then separated into 9 fractions by reversed-phase high-performance liquid chromatography. Two fractions displayed efficient radical-scavenging properties shown by Trolox equivalent antioxidant capacity assay. At least 347 peptides distributed in the different fractions were identified by tandem mass spectrometry. They mainly derived from the four different caseins, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), also called lactophorin, and peptido-glycan recognition protein-1. For the first time, cleavage sites were identified for these six proteins and the susceptibility of GlyCAM-1 towards bacterial proteolysis directly in milk was shown. Investigation of peptide sequences homologous to known bioactive peptides highlighted not less than 16 different putative biological activities. Fermentation of camel milk was thus a means of food processing to produce potential bioactive peptides.

1. Introduction

Antioxidant peptides present in food systems can play a vital role in the maintenance of antioxidant defence systems by preventing formation of, or scavenging, free radicals. Hence, the last two decades have seen growing interest in finding natural antioxidant peptides from food, because it is more and more clear that these peptides can prevent oxidative stress and delay the progress of many chronic diseases. Milk proteins are considered the most important source of bioactive peptides, and an increasing number of such peptides have been identified in milk protein hydrolysates and fermented dairy products (Abd El-Salam, El-Shibiny, 2013).

The search for bioactive peptides has mainly focused on bovine milk and, to a lesser extent, on ovine and caprine milk, but there is little information about biological activities of peptides produced in camel milk fermented by lactic acid bacteria (Shuangquan, Tsuda, Taku, 2008). Recently, fermentation of camel milk with a proteolytic strain of *Lactobacillus rhamnosus* has been performed and the angiotensin-converting enzyme (ACE)-inhibitory and antioxidant activities of the peptide fraction have been investigated (Moslehishad et al., 2013). In particular, increased proteolytic activity increased the free radical scavenging activity of the peptide fraction due to the generation of smaller breakdown products.

Caseins (CNs; α_{S1} -, α_{S2} -, β - and κ -CNs) represent the major protein fraction in camel milk and β -CN constitutes approximately 65% of total camel CNs versus 34% in cow (Kappeler, Farah, Puhan, 2003). The camel CNs are very susceptible to hydrolysis by purified gastro-intestinal proteases and their biological activities, such as antioxidant and ACE-inhibitory activities, are increased after enzymatic treatment (Jrad et al., 2014; Salami et al., 2011).

Free radical scavenging and antimicrobial properties of peptides generated by limited proteolysis of camel milk whey proteins have been investigated and the prevalence of α -lactalbumin-derived peptides in the improvement of the two types of activities after hydrolysis with trypsin, chymotrypsin, thermolysin, or proteinase K has been shown (Salami et al., 2010). Also, lactophorin I (LPcin-I), a peptide corresponding to the 113-135 carboxy-terminal region of lactophorin (also named bovine glycosylation-dependent cell adhesion molecule-1; GlyCAM-1), possesses an antibacterial activity against pathogen agents, whereas human red blood cells are not haemolysed in the presence of LPcin-I at concentrations less than 200 mM (Campagna, Mathot, Fleury, Girardet, Gaillard, 2004). However, to our knowledge, no study has found peptides from bovine or camel GlyCAM-1 or

has identified peptides from camel α -lactalbumin produced in fermented milk so far. Whey from camel milk contains other antimicrobial proteins involved in innate immunity, such as the heavy-chain antibodies Immunoglobulins G2 and G3, which are devoid of light chains, lactoferrin, and the peptidoglycan recognition protein-1 (PGRP-1; Sharma et al., 2011).

Fermentation of milk by proteolytic lactic acid bacteria is one of the economical and practical methods for the production of fermented dairy products enriched in bioactive peptides. The thermophilic lactic acid bacterium *Streptococcus thermophilus* is widely used as a starter to produce fermented milk products and to generate breakdown products from native proteins. The proteolytic system of *S. thermophilus* mainly consists of a cell wall protease named PrtS, and to a lesser extent, of an ABC-transporter of oligopeptides, a di/tripeptide transporter (Fernandez-Espla, Garault, Monnet, Rul, 2000), and also several cell wall-associated peptidases (Hafeez et al., 2015). It has been reported that proteolytic *S. thermophilus* strains generate peptides of various sizes from bovine individual CNs, among which some peptides contain potential bioactive sequences identified by mass spectrometry, and that PrtS is the main agent responsible for the hydrolysis of purified β -, α_{S1} - and α_{S2} -CNs (Chang et al., 2014; Miclo et al., 2012).

Until recently, most camel milk was used as food for shepherd families and for their animals; nowadays, the commercial production of camel milk is more developed. Therefore, it would be interesting to investigate the biological activities of peptides produced during the fermentation of camel milk with a proteolytic lactic acid bacterium such as *S. thermophilus* for which the proteolytic system is now well-documented. Generation of peptides by a proteolytic *S. thermophilus* strain acting on camel milk proteins either in milk or after their purification, has, however, not been studied so far. Moreover, bacterial hydrolysis of GlyCAM-1, in any species, has never been highlighted. The purpose of the present study was thus to liberate peptides from camel milk fermented with the proteolytic strain LMD-9 of *S. thermophilus*, and to investigate their free radical scavenging power. Each peptide was identified by mass spectrometry and compared with known bioactive peptides to investigate the potentiality of camel milk to unveil some biological activities after bacterial fermentation.

2. Material and Methods

2.1. Camel milk collection

Milk samples from 5 healthy camels (*Camelus dromedarius*) were collected. The animals, all belonging to experimental herd of the Livestock and Wildlife Laboratory (Institute of Arid Land, Medenine, Tunisia), were in the third month of lactation. Samples were collected manually in sterile bottles and the pH of the raw milks was checked at 25 °C and was approximately pH 6.6. The milk samples were mixed together and immediately stored at -20 °C until used.

2.2. Fermentation of camel milk

Experiments of production of peptides from natural fermentation of camel milk showed no detectable peptides derived from milk protein hydrolysis. Thus, the use of microorganisms selected for their proteolytic ability was shown to be necessary. The strain LMD-9 (ATCC BAA-491[®]) of *S. thermophilus* possesses a very active proteolytic system, mainly linked to the cell wall protease PrtS activity (Miclo et al., 2012) and was therefore used for the fermentation of camel milk.

The strain LMD-9 was stored at -80 °C in reconstituted skim milk (10%, w/v) until use. For the present study, the strain LMD-9 was grown overnight at 42 °C by inoculation of 50 mL of the stored culture into 5 mL of autoclaved skimmed camel milk (autoclaving at the typical 110 °C for 10 min). Then, 5 mL of the pre-culture were inoculated into 500 mL of autoclaved camel skimmed milk and incubated at 42 °C for 7 h (time needed to reach a pH value of 4.6). Below a pH value of approximately pH 5.0, the proteolytic system of *S. thermophilus* is inactive (Fernandez-Esplá et al., 2000) and the peptides produced were, therefore, not exposed to further degradation.

As a negative control, autoclaved camel skimmed milk was incubated at 42 °C overnight without inoculating any bacteria to check that the milk was actually sterile and that only trace amounts of peptides were already present in milk.

2.3. Preparation of camel milk peptides

A milk peptide fraction free of anionic and neutral components such as salts, lactic acid and lactose was prepared using a method derived from that of Farvin, Baron, Nielsen, and Jacobsen (2010). After fermentation for 7 h, the cultured milk was centrifuged for 25 min at 4000 x g at 20 °C. The supernatant was then adjusted to pH 2.0 with 2 M HCl to clot residual unhydrolysed camel CNs that hardly precipitate at pH 4.6, unlike bovine CNs, and also to protonate all the ionisable functions of the peptides. A new, clearer supernatant was then obtained after centrifugation for 25 min at 4000 x g at 20 °C and filtration through 0.45 µm filters. The supernatant was directly loaded onto a column (10 x 2.6 cm) of SE-Sephadex C-50[®] (Sigma-Aldrich, Saint-Quentin Fallavier, France) previously equilibrated with 0.01 M HCl. After washing with 200 mL of ultrapure water, the peptides were eluted in 200 mL of 2 M aqueous ammonia. The strong cation exchanger enabled to eliminate the uncharged and anionic compounds in the flow-through and wash fractions. The eluate was collected and freeze-dried to obtain a powder of peptides almost free of ammonia (24.7 mg mg⁻¹ of peptides; concentration determined with an ammonia assay kit purchased from Sigma-Aldrich). The total peptide fraction was solubilised at 10 mg mL⁻¹ in ultrapure water and subjected to centrifugal ultrafiltration at 3220 x g for 30 min at 20 °C using Amicon Ultra-15 Centrifugal Filter units with cut-off of 10 kDa (Merck Millipore, Darmstadt, Germany). After ultrafiltration, the retentate (approximately 1 mL) was diluted with 9 mL of ultrapure water and ultrafiltered again. The

centrifugal ultrafiltration was repeated and, finally, all the filtrates were pooled and freeze-dried as well as the retentates. A part of the pool of peptides lower than 10 kDa was subjected to additional centrifugal ultrafiltration at 3220 x g for 10 min at 20 °C using Amicon Ultra-0.5 Centrifugal Filter units with cut-off of 3 kDa. Two successive washes of the retentate (approximately 0.1 mL) were performed twice with 0.4 mL of ultrapure water. Finally, four fractions were obtained (>10, <10, 3-10, and <3 kDa) and were stored in a freeze-dried form at -20 °C until use.

2.4. Semi-preparative reversed phase-high performance liquid chromatography of peptides from fraction <10 kDa

Five hundred microlitres of the <10 kDa peptides (a solution at 10 mg mL⁻¹ in 5% acetonitrile in water, containing 0.1%, v/v, tri-fluoroacetic acid) were loaded into a semi-preparative LiChrospher C18 column (250 mm length x 10 mm inner diameter, 5 mm particle size, 10 nm porosity; Merck Millipore) connected to an Alliance 2690 HPLC unit (Waters, Milford, MA, USA) and equilibrated in 5% (v/v) acetonitrile in water, in the presence of 0.1% (v/v) trifluoro-acetic acid. Peptides were eluted at a flow-rate of 3 mL min⁻¹ using a linear gradient of 5-95% acetonitrile in water, in the presence of 0.1% (v/v) trifluoroacetic acid, between 5 and 95 min. The column temperature was maintained at 30 °C and detection was monitored between 200 and 300 nm using a photodiode array detector 996 (Waters). The reversed phase-high performance liquid chromatography (RP-HPLC) profiles were analysed with the Millennium 32 software version 3.05.01 (Waters). A first fraction (noted F1) was collected and corresponded to materials eluted during the first 5 min and then, other fractions (F2-F9) were collected every 10 min. Each chromatographic fraction was finally freeze-dried.

2.5. Free radical scavenging activity of the peptide fractions

The free radical scavenging activity of peptides was assessed according to the Trolox equivalent antioxidant capacity (TEAC) method as previously described (Jrad et al., 2014). Briefly, scavenging of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS^{•+}) by peptides at 30 °C for 10 min was monitored by an absorbance decrease at 740 nm. The concentration of peptides able to transform 50% of ABTS^{•+} to ABTS (IC₅₀) was expressed in mg L⁻¹ determined by three measurements (mean ± standard deviation; SD) and the TEAC was expressed in mM TE (Trolox equivalent; mean ± SD). The peptide concentration was determined in triplicate by the o-phthalaldehyde method using tryptone as standard according to Salami et al. (2010) and the ammonia content, however low, was systematically deduced in the case of peptides that were not separated by RP-HPLC (o-phthalaldehyde reacts with α-NH₂ and ε-NH₂ groups but also with ammonia).

2.6. Nano-electrospray ionisation tandem mass spectrometry coupled with liquid chromatography (HPLC/nESI-MS/MS)

Peptides of each RP-HPLC fraction were identified by on-line nano-electrospray ionisation tandem mass spectrometry coupled with liquid chromatography (HPLC/nESI-MS/MS) on a nanoLC Dionex RSLC[®] system coupled to a ThermoScientific Q Exactive Quadrupole-Orbitrap mass spectrometer (Dionex/Thermo-Scientific, Villebon-sur-Yvette, France) as previously described (Jrad et al., 2014). Sequence alignments with known peptides displaying any biological activity were performed with the PepBank database available on <http://pepbank.mgh.harvard.edu/search/blast>.

3. Results and discussion

3.1. Peptide size-dependent free radical scavenging activity

Table 1 : Free radical scavenging activity of peptides fractionated according to size

Peptides	TEAC (mM TE)	IC ₅₀ (mg L ⁻¹)
TP	1925 ± 90	13 ± 1
>10 kDa	817 ± 60	33 ± 2
3-10 kDa	525 ± 60	96 ± 5
<3 kDa	1371 ± 90	20 ± 1

a Abbreviations are: TP, total peptides; TEAC, Trolox equivalent activity capacity; IC₅₀, concentration of peptides needed to scavenge 50% of free radicals. Data are the mean of three experiments ± SD.

First investigations focused on the free radical scavenging properties of peptides fractionated according to molecular mass (Table 1). The total peptides displayed a good potential of free radical scavenging and this potential was mainly recovered in the <3 kDa peptides.

The present result was consistent with reported studies performed with bovine peptides generated by bacteria from purified CNs (Chang et al., 2013) or milk proteins (Farvin et al., 2010), showing that the free radical scavenging activity of fermented bovine milk is due to peptides of size < 3 kDa. However, in the case of camel milk fermented with *Lb. rhamnosus*, the free radical scavenging activity is rather found for larger peptides, i.e.,

5-10 kDa (Moslehishad et al., 2013). Interestingly, the free radical scavenging power of the <3 kDa peptides (1371 mM TE) was similar to that of the 5-10 kDa peptides generated by *Lb. rhamnosus* (close to 1000 mM TE; Moslehishad et al., 2013). However, the size difference observed between the two studies (<3 kDa versus 5-10 kDa) was probably due to the kind of the bacterial genus used. Thus, the nature of the peptides produced and, therefore, their potential free radical scavenging efficiency depended on the specificity of the proteolytic system of the bacteria used. This was in agreement with another study showing that the free radical scavenging power of camel caseinopeptides depends both on the digestive protease selected (trypsin, chymotrypsin, or pepsin) and on the size of the peptides (Salami et al., 2011). Nevertheless, the free radical scavenging power of these peptides generated by in vitro CN digestion is less efficient than that of the peptides produced by fermentation of camel milk with *Lb. rhamnosus* (Moslehishad et al., 2013). In a similar manner, it was thus not surprising that the TEAC of the total peptides produced by *S. thermophilus* in the present study (1925 mM TE) was higher than that of camel milk hydrolysate obtained by combined action of pepsin and pancreatin (437 mM TE) in previous work (Jrad et al., 2014).

3.2. Peptide identification and source proteins

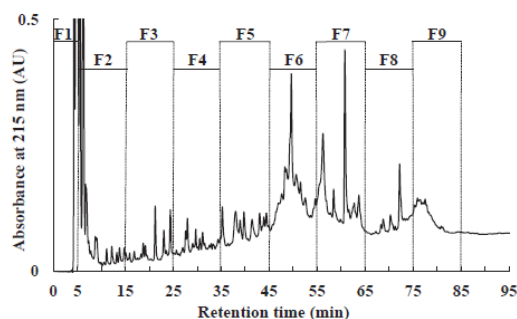


Fig. 1. Semi-preparative RP-HPLC separation of <10 kDa peptides extracted from fermented camel milk. F1-F9: fractions collected, A.U., absorbance unit.

To further explore the free radical scavenging of the camel peptides and to identify the most peptides exhibiting potential biological activity, the peptides <10 kDa were separated by RP-HPLC (Fig. 1). The peptides <10 kDa were separated into 8 eluted fractions, F2-F9, whereas the unadsorbed fraction F1 did not contain any identifiable peptides as revealed by HPLC/nESI-MS/MS analysis. To be identified, a peptide needs to contain more than five residues for specificity reasons and also needs to be smaller than approximately 5 kDa to be efficiently fragmented by tandem mass spectrometry. As a result, a total of 347 peptides were identified in all fractions. Most of the peptides derived from the four individual CNs, especially from β -CN, the major component (Fig. 2). Also, it was noteworthy that peptides were generated from soluble proteins specific to camel whey, such as PGRP-1, not found in bovine milk, and GlyCAM-1. The latter is in fact a minor component in milk of most of the species studied (approximately 300 mg L⁻¹ in bovine milk), but its concentration is much more higher in camel milk and ranges from approximately 1 g L⁻¹ to approximately 5 g L⁻¹ (Abd El-Salam & El-Shibiny, 2013). Interestingly, no peptide derived from α -lactalbumin or lactoferrin was found. The strong resistance of these globular, compact proteins towards bacterial proteolysis was probably the reason.

The camel CNs have low homology with bovine CNs, being 60.3% for α_{S1} -CN, 73.0% for α_{S2} -CN, 76.0% for k-CN, and 80.4% for β -CN (Kappeler et al., 2003). Consequently, the susceptibility towards proteolysis of the camel CNs and the type and concentration of peptides generated, particularly the bioactive peptides, might be different from that of bovine CNs. Indeed, and unlike the bovine β -CN, α_{S1} -CN, and α_{S2} -CN (Miclo et al., 2012), the entire sequences of the camel counterparts displayed cleavage sites for the proteolytic system of *S. thermophilus* LMD-9 leading to the generation of many peptides. In the present study, 94 cleavage sites and 144 peptides were found for β -CN, 65 cleavage sites and 94 peptides for α_{S1} -CN, and 25 cleavage sites and 24 peptides for α_{S2} -CN were found.

Similarly, a better digestibility towards gastro-intestinal enzymes of the purified camel CNs over the bovine milk proteins has been already observed (Salami et al., 2011). The camel CN sequences are thus more accessible to proteases than the bovine ones. According to Sadat-Mekmene et al. (2011), the bovine CNs, assembled into micelles in milk, are more resistant to proteolysis by strains of *Lactobacillus helveticus* or *Lactobacillus delbrueckii* subsp. *lactis* grown in milk than sodium caseinate submitted to bacterial hydrolysis. Indeed, Sadat-Mekmene et al. (2011) have observed a drastic reduction in the number of peptides when proteolysis occurs in milk rather than with purified CNs. However, in the case of camel milk, the micelles seemed to have a looser structure, as the individual CNs were readily accessible to attack by the proteolytic system of LMD-9.

The hydrophilic N-terminal region of bovine β -CN is resistant toward hydrolysis, whereas the C-terminal region is more accessible to the proteolytic system of *S. thermophilus* LMD-9 (Chang et al., 2014; Miclo et al., 2012), but also to those of *Bifidobacterium longum* (Chang et al., 2013) and *Lb. helveticus* or *Lb. delbrueckii* subsp. *lactis* (Sadat-Mekmene et al., 2011). This was, however, not the case for camel β -CN, for which the identified peptides covered the entire sequence. Similarly, the sequences of camel α_{S1} -CN and α_{S2} -CN were almost reconstituted from the identified peptides, except the tetraphosphorylated (4P) regions α_{S1} -CN-4P (70-78) and α_{S2} -CN-4P (108-123) and the hydrophobic regions α_{S1} -CN (168-187) and α_{S2} -CN (143-167). When the degree of phosphorylation was high, the caseinophosphopeptides (CPPs) might precipitate during the preparation step by forming a network stabilised by calcium phosphate cross-linkages at basic pH, and therefore, not be recovered in the peptide fraction. Also, the two hydrophobic regions might co-precipitate, but rather during the acidification according to another physico-chemical mechanism.

It is reported that bovine α_{S1} -CN monomers are able to form noncovalent multimers that could protect the protein against hydrolysis (Miclo et al., 2012). To date, it is however not clear if the good accessibility of camel α_{S1} -CN was due or not to the absence of polymerisation. Camel α_{S1} -CN was prone to hydrolysis by *S. thermophilus* when grown in milk. In particular, the region 41-91 reported to be resistant toward bacterial hydrolysis in the bovine species (Chang et al., 2013, 2014; Miclo et al., 2012) was cleaved into numerous fragments in the case of the camel protein (24 cleavage sites). Camel α_{S1} -CN was more readily hydrolysed than the bovine counterpart submitted to the action of *B. longum* (no peptide found; Chang et al., 2013) or by *Lb.* strains (between 1 and 7 peptides found according to the strain used; Sadat-Mekmene et al., 2011). In the case of purified bovine α_{S1} -CN, 89 peptides are generated by *S. thermophilus* LMD-9, mainly from the C-terminal domain of the protein, but the N-terminal 1-88 region is almost not hydrolysed (only 2 cleavage sites; Miclo et al., 2012). Bovine α_{S1} -CN molecules form tetramers in phosphate buffer (pH 8.1) that partly hinder the accessibility to the enzymes. The accessibility of potential cleavage sites is a determinant condition for the susceptibility to hydrolysis, more important than the hydrophilic/hydrophobic balance (Miclo et al., 2012). A number of 25 cleavage sites were found for the camel α_{S2} -CN, but the C-terminal domain was hydrolysed to a lesser extent than the bovine one. It is hypothesised that α_{S2} -CN might be more closely linked to the micelle in camel milk than in bovine milk and therefore less accessible.

Regarding the sequence of k-CN, 28 fragments were found (and 18 cleavage sites), all located in the N-terminal region 1-75. This was much more than the two peptides found after treatment of bovine sodium caseinate with *B. longum* (Chang et al., 2013) or the absence of peptides observed when *Lb.* strains were grown in bovine milk (Sadat-Mekmene et al., 2011), suggesting that the camel k-CN was also prone to proteolysis. No results, however, have been reported about the susceptibility of bovine k-CN toward the proteolytic system of *S. thermophilus* during milk fermentation. On the other hand, no fragment of the 76-162 C-terminal region was highlighted in the peptides <10 kDa, probably because the MS experimental conditions used in the present study were not suitable for identifying any glycopeptides. The C-terminal region carries glycan chains potentially linked at Thr134, Ser141, and Thr158 (by similarity to the bovine sequence), indeed. It was noteworthy that three peptides containing Cys10 in reduced state were found (1-17, 1-18, and 1-32), suggesting that disulphide linkage involving Cys10 and Cys76 was not, or only partly, formed during the folding of the structure of k-CN. In the bovine species,

native k-CN was always found in an oxidised form. This might explain why some authors have not found any Cys-containing peptides generated from the N- and C-terminal moieties of bovine k-CN (Chang et al., 2013). Nevertheless, Chang et al. (2014) have shown that a soluble form of PrtS released in the medium by the 4F44 strain of *S. thermophilus* is able to hydrolyse bovine k-CN in purified sodium caseinate (9 peptides found).

A specificity of camel milk was that two whey proteins were sensitive toward the proteolysis of *S. thermophilus* grown in milk. Mainly, GlyCAM-1 was hydrolysed by the proteolytic system of the bacterium (34 cleavage sites and 54 peptides produced). The inner region 44-121 was the most sensitive, but a few peptides corresponded to the N- and C-terminal regions. The region 25-43 was not recovered, probably because it can contain 4 phosphate groups at Ser30, Ser35, Ser39, and Ser41, all located in SXE or SXS (S for phosphoserine) consensus sequences of phosphorylation by casein kinase I. The region 122-127 was also not found, probably because it might be hydrolysed into smaller peptides (<5 residues) not identifiable by the MS/MS database searches used in this study. Sharma et al. (2011) have shown that, in the PGRP family, only the camel PGRP-1 forms noncovalent homotetramers. The tridimensional structure of each monomer includes a central β -sheet surrounded by three α -helices and is stabilised by three disulphide linkages, Cys7-Cys131, Cys23-Cys68, and Cys44-Cys50. The monomer is ended by a C-terminal tail (domain 166-172) protruding into aqueous solvent. In the quaternary structure, four monomers are self-associated in a tightly packed tetramer, whereas the four C-terminal tails were accessible to the solvent. After milk fermentation, only 3 peptides were identified, which corresponded to sequences located in the C-terminal region 141-165 of PGRP-1. The main part of the protein (region 1-140) involved in the tetramer formation was likely not accessible to the proteolytic system of LMD-9. It was noteworthy that the C-terminal tail was released by bacterial proteolysis, as the peptide bond 165-166 was cleaved. The C-terminal peptide PGRP-1 (166-172) was, however, not found, probably because it was further hydrolysed into smaller fragments which were not detectable.

3.3. Free radical scavenging activity of peptides separated by RP-HPLC

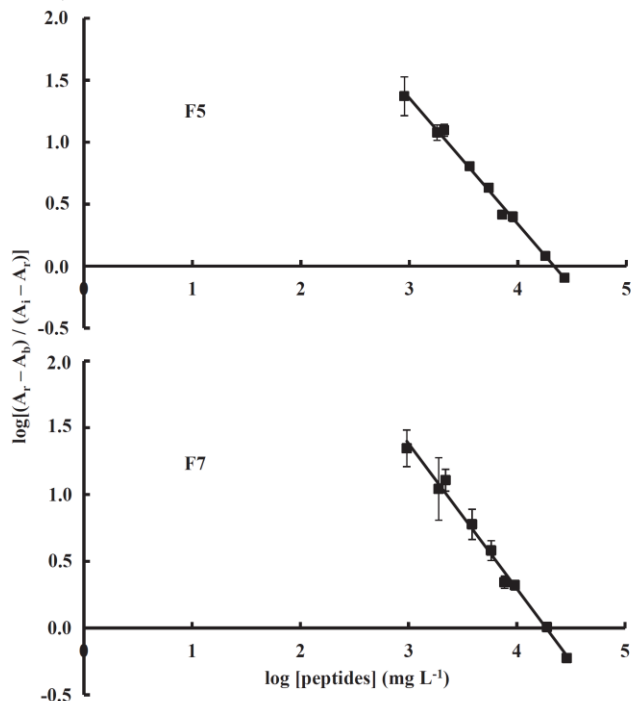


Fig. 3 : Free radical scavenging activity of peptides of F5 and F7 as a function of concentration. The IC₅₀ is the x-intercept of the linear representation. A_i, absorbance of the initial ABTS^{••} radical; A_r, absorbance of the remaining radical; A_b, absorbance of the phosphate buffer (A_b = 0.05). The assays were conducted in triplicate and vertical bars indicate mean values \pm SD.

To date, no antioxidant peptides have been identified in fermented camel milk. As Moslehi et al. (2013) have shown that peptides with molecular masses > 3 kDa exhibit highly efficient antioxidant properties, all the peptides < 10 kDa, and not only the small peptides < 3 kDa, were taken into account in the present investigation and were fractionated by RP-

HPLC (Fig. 1). The chromatographic fractions could be divided in two groups; F5 and F7 regrouped the strongly antioxidant peptides (SAPs) having respective IC₅₀ values of 21 and 19 mg L⁻¹ (Fig. 3) and TEAC values of 1604 μ M TE and 1662 μ M TE, whereas the other fractions F2, F3, F4, F6, F8, and F9 were gathered in a group constituted by the weakly antioxidant peptides (WAPs) having TEAC ranged from 350 to 933 μ M TE (Table 2).

Table 2 : Free radical scavenging activity of RP-HPLC fractions (F2eF9) of the peptides < 10 kDa.^a

Peptides	TEAC (μ M TE)	IC ₅₀ (mg L ⁻¹)
F2	525 \pm 60	137 \pm 14
F3	933 \pm 90	45 \pm 3
F4	729 \pm 9	69 \pm 7
F5	1604 \pm 60	21 \pm 1
F6	904 \pm 90	58 \pm 3
F7	1662 \pm 90	19 \pm 1
F8	437 \pm 30	41 \pm 3
F9	350 \pm 30	86 \pm 3

Abbreviations are: TEAC, Trolox equivalent activity capacity; IC₅₀, concentration of peptides needed to scavenge 50% of free radicals. Data are the mean of three experiments \pm SD.

The SAPs derived from the six proteins: β -CN (78 peptides in F5 and F7), α _{S1}-CN (50 peptides), κ -CN (24 peptides), α _{S2}-CN (9 peptides), GlyCAM-1 (31 peptides), and PGRP-1 (3 peptides). Among the SAPs inherent to β -CN, five peptides (Ser165-Glu183, Ser165-Arg184, Ser165-Val188, Ser167-Val188, and Val171-Val188) might possess free radical scavenging activity as they share seven residues (KVLVPVQ) with the bovine β -CN antioxidant peptide KVLVPVQK (Rival, Boeriu, & Wichers, 2001). In addition, four of these five peptides also contained another encrypted sequence, VPYPQR, that is also common to the free radical scavenger AVYPYQR from bovine β -CN (Rival et al., 2001). In addition, 17 peptides derived from the 193-217 C-terminal region of camel β -CN and eluted in the SAP fractions, contained the sequence PVRG, which is also included in the β -CN bovine antioxidant peptide GPVRGPFPII (Hernandez-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005). As most of the SAPs derived from camel β -CN, they might play a crucial role in the free radical scavenging properties of the fractions F5 and F7. However, the role of other peptides generated from the other proteins should not be excluded. Bacterial hydrolysis of camel α _{S1}-CN released a C-terminal peptide G197GKTDVMPQWW217, that has the possibility of possessing two successive Trp residues at its carboxyl extremity and might therefore possess remarkable free radical scavenging properties, as it is already observed with synthetic peptides (Tsopmo et al., 2011). A similar finding might be made with the C-terminal M168TPWNHIKRYF178 peptide of camel α _{S2}-CN, which contains three aromatic residues (Trp171, Tyr177, and Phe178) two of which are located at the penultimate and final positions. To date, no data exists about the antioxidant potential of peptides released from the other milk proteins κ -CN, GlyCAM-1 and PGRP-1. It was noteworthy that most of the peptides produced from GlyCAM-1 (57.4% of the peptides) belonged to the SAP group and might play a significant role in the free radical scavenging properties.

3.4. Other potential biological activities

By analogy to known bioactive peptides, a variety of potential biological properties attributed to camel peptides is listed in Table 3. At least 16 different biological activities were potentially carried by many peptides. Some of these peptides would be also precursors of smaller bioactive fragments released by gastro-intestinal digestion and more readily absorbed through the intestinal barrier. However, large breakdown products might be resistant toward gastrointestinal enzymes in the same way as bovine CPPs.

No study has reported the isolation and characterisation of CPPs in the digest of camel CN or fermented camel milk. Abd El-Salam and El-Shibiny (2013) suggest that camel CPPs could have antioxidant properties, because the primary structures of the camel β -CN, α _{S1}-CN, and α _{S2}-CN show the presence of phosphate clusters (SSSEE) comparable with that of the corresponding bovine milk CNs. The camel CPPs β -CN S15ISSSEESITHINK28 and α _{S2}-CN K1HEMDQGSSEESINVSQQKFK22 were recovered among the WAPs and might contribute to the antioxidant activity of fermented camel milk by preventing lipid oxidation (Meisel, 2004). However, no CPP containing

Table 1: Peptides from fermented camel milk recovered in the RP-HPLC fractions and having at least five identical residues (bold characters) with known bioactive peptides.^a

Potentially bioactive peptides	HPLC fraction	Potentially bioactive sequence	Homologous bioactive peptides	Biological activity	Reference
Camel β -CN peptides: 15-28	F4	Camel β -CN-4P (15-28): Σ 1 $\Sigma\Sigma\Sigma$ EESITHINK	Bovine β -CN-4P (1-25): $-\Sigma$ 1 $\Sigma\Sigma\Sigma$ EESETRINK	Antioxidant, mineral binding, immunomodulatory, cytomodulatory	Meisel (2004)
29-74, 34-74, 35-74, 36-74, 40-74, 41-74	F7	Camel β -CN (59-68):	Bovine β -CN (58-76):	Antihypertensive	Quiros, del Mar Contreras, Ramos, Amigo, and Recio
42-74, 43-74, 44-74, 45-74, 47-74, 48-74	F7	LVYSHTEPIP	LVYFPFGPIPNLQNIIP		
49-75, 50-75, 52-74, 57-74, 58-74	F7				
29-74, 34-74, 35-74, 36-74, 40-74, 41-74	F7	Camel β -CN (65-69):	Human β -CN (54-59):	Immunostimulatory	Parker et al. (1984)
42-74, 43-74, 44-74, 45-74, 47-74, 48-74	F7	EPIPY	VEPIPY		
49-75, 50-75, 52-74, 57-74, 58-74, 64-74	F7				
75-83	F7	Camel β -CN (77-79):	Bovine β -CN (74-76) & (84-86):	Antihypertensive	Boelsma and Kloek (2009)
77-82	F5	LPP	IPP & VPP	Atherosclerosis prevention	Nakamura et al. (2013)
84-94	F7	Camel β -CN (84-91):	Bovine β -CN (80-90):	Antihypertensive	Abubakar, Saito, Kitazawa, Itoh (1998)
		VMVPELQP	TPVVVPPFLQP		
78-102	F8	Camel β -CN (82-91):			
60-92, 62-92, 76-94, 78-96	F9	PAVMVPELQP			
101-124, 107-124	F7	Camel β -CN (105-124): PKRKEMPLLQSPVVPFTESQ	Bovine β -CN (94-123): -PKHKEMPFPKYPVEPFTESQ	Intestinal protection	Plaisancie et al. (2015)
101-120, 101-121, 101-124	F7	Camel β -CN (106-117): KRKEMPLLQSPV	Bovine β -CN (105-117): KHKEMPFPKYPVE	Cytomodulatory	Nagaune et al. (1989)
107-124	F7	Camel β -CN (115-122): SPVVPFTE	Bovine β -CN (114-121): YPVQPFTE	BPP (antihypertensive)	Lebrun et al. (1995)
131-142, 133-142, 134-144	F8	Camel β -CN (134-140):	Bovine β -CN (133-138/139):	Antihypertensive	Quiros et al. (2009)
131-145	F9	LHLPLPL	LHLPLPL		
170-188, 171-188, 171-189	F6	Camel β -CN (170-176):	Bovine β -CN (169-174/175):	Antihypertensive	Maeno, Yamamoto, and Takano
165-183, 165-184, 165-188	F7	KVLPVPQ	KVLPVPQ		
163-183, 163-184, 165-200, 171-194, 171-	F8		Bovine β -CN (169/170-176): KVLPVPQK	Antioxidant	Rival et al. (2001)
170-188, 171-188, 171-189	F6	Camel β -CN (179-184):	Bovine β -CN (177-183):	Antioxidant & antihypertensive	Rival et al. (2001)
165-184, 165-188	F7	VPYPQR	AVPYPQR		Maeno et al. (1996)
163-184, 165-200, 171-194, 171-217	F8				
171-217, 183-217, 184-217, 185-216	F8	Camel β -CN (186-203): MPVQAVLFPQEPVPDPVR	Bovine β -CN (184-202) DMPIQAFLLYQEPVGLPVR	Anti-inflammatory	Malinowski et al. (2014)
193-216, 193-217, 194-217	F7	Camel β -CN (195-207): QEPVPDPVRLHP	Bovine β -CN (193-209): YQEPVGLPVRGPFPIIV	Immunomodulatory Mitogenic	Sandre et al. (2001) Coste et al. (1992)
195-215, 195-216, 195-217	F7				
183-217, 184-217, 185-216, 186-217	F8				
187-217, 188-217, 189-217, 191-217	F8				
193-216, 193-217, 194-217, 195-205, 195-	F7	Camel β -CN (198-207):	Bovine β -CN (197-206):	Antihypertensive	Quiros et al. (2009)
195-216, 195-217, 196-217, 197-217, 198-	F7	VPDPVRLHP	VLGPVRGPF		
195-217,197-217	F8				
193-216, 193-217, 194-217, 195-205, 195-	F7	Camel β -CN (201-207):	Bovine β -CN (199-208):	Antioxidant	Hernandez-Ledesma et al.
195-216, 195-217, 196-217, 197-217, 198-	F7	PVRGLHP	GPVRGPFPII		
195-217,197-217	F8				
Camel α _{s1} -CN peptides					
41-55, 42-57	F4	Camel α _{s1} -CN (47-53): LKDTRNE	<i>P. falciparum</i> EBA-175 peptide: DRNSNTLHLKDYRNEENERH	Red blood cell binding	Rodríguez et al. (2000)
42-60, 42-67	F5	Camel α _{s1} -CN (47-58):			
41-60	F6	LKDTRNEPTEDH			
Camel κ -CN peptides					
19-37	F5	Camel κ -CN (25-34):	Bovine κ -CN (25-34):	Casoxin C (opioid antagonist)	Takahashi et al. (1997)
16-37, 16-41, 18-37, 18-41, 19-41, 20-37	F7	YFPIQFVQSR	YIPIQYVLSR		
33-47	F6	Camel κ -CN (35-41): YPSYGIN	Bovine κ -CN (35-41): YPSYGLN	Casoxin A (opioid antagonist)	Chiba et al. (1989)

Potentially bioactive peptides	HPLC fraction	Potentially bioactive sequence	Homologous bioactive peptides	Biological activity	Reference
Camel α s 2-CN peptides 1-22	F4	Camel α s 2-CN-3P (1-22): KHEMDQGGΣΣΣEESINVSQQKFK	Bovine α s 2-CN-4P (1-32): KNTMEHVΣΣΣEESIISQETYSQEKNNMAINPSK	Antioxidant, mineral binding, immunomodulatory	Meisel (2004)
59-72	F7	Camel α s 2-CN (61-68): KISQFYQK	Bovine α s 2-CN (164-179): LKKISQRYQKFALPQY	Calmodulin antagonist	Kizawa, Naganuma, and Murakami (1996)
79-93, 79-94	F5	Camel α s 2-CN (81-90):	Bovine α s 2-CN (102-111):	Food allergy prevention	Tanabe et al. (2006)
77-94, 79-92	F6	GQIVMNPWDQ	GPIVLNPWDQ		
71-93	F7				
Camel PGRP-1 peptides 141-161, 146-161	F5	Camel PGLYRP1 (147-154):	Human endostatin (1-12-NH ₂):	Angiogenesis inhibition	Kolozsi, Jancso, Nagy, and Gajda (2009)
146-165	F7	HRDVQPTL	HSHRDFQPVLHL-NH₂	& antitumoral	

^a The antihypertensive peptides, but not the in vitro angiotensin-converting enzyme inhibitors, are listed in the Table. Abbreviations are: CN, casein; Σ , phosphoserine residue; P, phosphate group; BPP, bradykinin-potentiating peptide; *P. falciparum* EBA-175, *Plasmodium falciparum* erythrocyte-binding antigen 175; PGRP-1, peptidoglycan recognition protein 1. Underlined sequences indicate sequences LPP and VPP close to the bovine LPP and VPP, and encrypted in camel peptides that might exhibit potential antihypertensive and anti-inflammatory activities. The sequence underlined twice corresponds to b-casokinin-10, a cytomodulatory decapeptide conferring the mitogenic effect of β -CN (193e209).

the 68-75 sequence of camel α_{S1} -CN was found in the peptide fraction <10 kDa, but it was possible that a CPP might be generated from α_{S1} -CN in fermented milk, not be recovered in the peptide fraction, and act as metal ion chelator. The phosphate cluster of camel α_{S1} -CN possesses an extra phosphoserine residue (S68GSSSSEE75) conferring to camel α_{S1} -CN CPP different metalbinding capacity than its bovine counterpart (Abd El-Salam & El-Shibiny, 2013).

On the other hand, the bovine CPPs β -CN-4P (1-25), α_{S1} -CN-5P (59-79), and α_{S2} -CN-3P (1-32) have multifunctional activities, such as prevention of lipid oxidation, mineral binding, and immunomodulatory properties. In addition, β -CN-4P (1-25) also possesses cytomodulatory property (Meisel, 2004). Similarly, the camel CPPs β -CN-4P (15-28) and α_{S2} -CN-3P (1-22) and potential CPPs containing the 68-75 region of the camel α_{S1} -CN generated in fermented camel milk might also have such multifunctional effects.

Regarding the camel GlyCAM-1, no data are available about the potential role of the tetraphosphorylated region (25-43 region). Nevertheless, it is reported that the phosphorylated region of the bovine GlyCAM-1 confers to the protein a mineral-binding capability as efficient as that of bovine CNs, despite the absence of a phosphate cluster (Bernos, Girardet, Humbert, & Linden, 1997).

Many potential bioactive peptides, all from β -CN, might be inhibitors of ACE in vitro or have hypotensive effect in vivo. This activity is well-documented in the literature and only the in vivo studies showing antihypertensive effects have been listed in Table 2. Camel milk fermented by *Lb. rhamnosus* displays ACE-inhibitory properties higher than those of fermented bovine milk (Moslehi et al., 2013). It was therefore not surprising that the camel milk fermented by *S. thermophilus* might contain peptides exhibiting hypotensive effect. It was worthy to note that sequences L76PP78, V118PF120, and I158PP160 that are encrypted in camel β -CN peptides generated in fermented milk might exhibit potential antihypertensive and atherosclerosis prevention activities after their possible release by gastro-intestinal digestion, similarly to the bovine lactotriptides IPP and VPP (Boelsma & Kloek, 2009; Nakamura et al., 2013). It was also interesting to note that fermentation of camel milk produced β -CN-derived peptides 171-217, 183-217, 184-217, and 185-216 that contain the region 185-203, AMPVQAVLPFQEPVDPVPR, having potential anti-inflammatory properties. Indeed, the bovine homologous peptide DMPIQAFLLYQEPVLPVPR, produced by action of trypsin, is considered as the first CN-derived peptide identified that exerts an anti-inflammatory effect in vitro (Malinowski, Klempt, Clawin-Radecker,

Lorenzen, & Meisel, 2014). In the case of fermented camel milk, sequence 185-203 could be released in the gut during the post-prandial phase by digestive enzymes such as trypsin and reveal its potential anti-inflammatory action. In addition, these four peptides appeared to be multifunctional, because they also contained (as well as eight other identified peptides), a casokinin-like sequence (194-203 region) that

might display ACE-inhibitory activity, as does its bovine counterpart (Meisel, 2004).

Camel κ -CN released several peptides that might be opioid antagonists homologous to casoxins A and C (Chiba, Tani, & Yoshikawa, 1989; Takahashi et al., 1997). In particular, the sequence of camel κ -CN (35-41) is almost identical to the bovine casoxin C (YPSYGIN versus YPSYGLN, respectively) and might therefore belong to the casomorphin group, like the camel casoxin C-like peptide. However, the camel κ -CN-derived peptide 107-115 identified in camel milk fermented by *Lb. helveticus* as ACE-inhibitory peptide (Shuangquan et al., 2008) was not found in the present work. Similarly, no camel κ -CN-derived peptide including the anti-hypertensive I100PP102 sequence was found. Hydrolysis of purified bovine α_{S1} -CN by the proteolytic system of LMD-9 can generate the antibacterial isracidin (fragment 1-23; Miclo et al., 2012). Fermentation of camel milk by *S. thermophilus* LMD-9 produced similar peptides corresponding to the 1-23 and 1-25 regions of the camel α_{S1} -CN. These peptides might possess antibacterial property against pathogens and consequently, would correspond to isracidin-like peptides. However, the camel 1-23 peptide presents very low homology with isracidin (only 5 identical residues over 23). Moreover, another major difference is that the camel sequence carries a phosphoserine residue (S18), whereas isracidin is not phosphorylated.

The bovine GlyCAM-1 C-terminal domain is able to form a large amphipathic α -helix involving 38 residues (Girardet & Linden, 1996) that confers to the corresponding peptide LPcin-I antibacterial properties (Campagna et al., 2004). Despite low sequence homology, the camel protein may be also structured into a C-terminal amphipathic α -helix involving approximately 35 residues (Girardet & Linden, 1996) and large peptides, such as the 77-121 fragment produced, might exert an antimicrobial action. In this last case, investigation of secondary structures was more relevant than the comparison of primary structures in order to highlight antibacterial sequences. Nevertheless, no peptide corresponding to the whole amphipathic domain was found. It will thus be interesting to investigate if the 1-23 and 1-25 peptides of camel α_{S1} -CN and the 77-121 peptide of camel GlyCAM-1 possess any antimicrobial activity.

Abd El-Salam and El-Shibiny (2013) suggest that PGRP-1 could be a source of antibacterial peptides. Not found in bovine milk, but present in camel milk in higher concentrations than the other known antibacterial proteins (lactoferrin, lactoperoxidase, or lysozyme), PGRP-1 can bind to peptidoglycan structures in the cell wall and inhibit the growth of pathogens. This protein is multifunctional, because it can bind to lipopolysaccharide from Gram-negative bacteria and to lipoteichoic acid from Gram-positive bacteria and, consequently, inhibit expression of inducible pro-inflammatory cytokines (Sharma et al., 2011). This whey protein was, however, resistant toward proteolysis, as only few peptides corresponding to the C-terminal domain were produced. The C-terminal tails of each PGRP-1 monomer associated in tetramers are not involved in the binding to the bacterial toxicants. It is thus thought that the C-terminal peptides did not have anti-inflammatory effect as described.

4. Conclusion

The present study has shown that the four camel CN components, and also whey proteins such as GlyCAM-1 and PGRP-1, were susceptible to hydrolysis by the proteolytic system of *S. thermophilus* grown in camel milk. For the first time, cleavage sites of these camel proteins have been identified and peptides with potential biological activity have been suggested. In particular, free radical scavenging activity related to some identified small peptides (<3 kDa) was experimentally shown. The strain LMD-9, which is widely used in dairy product manufacture, could be used for the release of bioactive peptides during processing. Some of the peptides showed marked structural similarities with previously identified antihypertensive, immunomodulatory, anti-inflammatory, and antioxidant peptides and may be used as multifunctional ingredients of functional foods to control cardiovascular diseases. Further studies are necessary and will be performed to validate the biological effects of the fermented milk and to study the stability of the bioactive peptides during the storage of the products and their bioavailability for the consumer.

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