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ANTIMICROBIAL ACTIVITY OF CAMEL MILK CASEIN AND ITS HYDROLYSATES

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The aim of this study was to evaluate the antimicrobial activity of camel caseins and their hydrolysates by gastro-intestinal proteolytic enzymes against 3 Gram-positive and 2 Gram-negative bacterial strains. Camel caseins (CN) were hydrolysed by successive action of pepsin and pancreatin. Hydrolysis of CN was checked by electrophoresis and gel filtration chromatography (GFC). Both techniques showed that CN was hydrolysed into peptides. Among the tested bacteria, a decrease of 19.3‰±0.02 of *E. coli* XL1 blue cells growth was observed in the presence of undigested camel casein at a concentration of 20 mg ml^{-1}. After successive hydrolyses by pepsin and pancreatin, camel milk casein hydrolysates still exhibited anti-bacterial activity against *E. coli* XL1 blue strain (19.73±0.01% growth inhibition under the same conditions). Gram-positive strain growth was not affected by intact camel CN, while, at the same concentration (20 mg ml^{-1}), their hydrolysates slightly inhibited the growth of these bacteria. This suggests that antibacterial peptidic fragments of caseins were generated by pepsin and pancreatin.

Keywords: camel casein, pepsin, pancreatin, antibacterial activity

The protein content of camel milk differs markedly from cow milk, although the caseins content in camel milk is slightly higher. However, β-CN is the major protein in camel milk (65% of total CN), and the α_{s1}-CN level (22%) is low, while β-CN and α_{S1}-CN are almost equally abundant in cow milk (37 and 30% of total caseins, respectively). The proportion of κ-CN in camel milk is only 3% (vs. 10–12% in cow milk, Kappeler et al., 1998). On the other hand, cow caseins were reported to be more susceptible to hydrolysis by trypsin than camel milk CN, whereas camel CNs were more readily hydrolysed by chymotrypsin (Salami et al., 2008). This suggests that bioactive peptides released following enzymatic hydrolysis of camel milk caseins may be different from those derived from bovine caseins.

It is now well known that beside the main physiological role of milk CN as a source of amino acids required for the growth of neonate, these proteins may contain peptides encrypted in their sequences, which can exhibit various biological activities once released. Such peptides can be released in vivo upon digestion, in vitro by enzymatic hydrolysis, or during fermentation as a result of the action of proteases produced by lactic acid bacteria. CN hydrolysates and CN-derived bioactive peptides showed several biological activities (Silva & Malcata, 2005).

The presence of antimicrobial activity in the bovine, ovine, and buffalo α_{s1}-, α_{s2}- and κ-casein hydrolysates has been reported (Zucht et al., 1995; Recio & Visser, 1999; López-

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Expósito et al., 2006). Several antimicrobial peptides have been identified from bovine casein hydrolysates, mainly isracidin and caseicin (Lahov & Regelson, 1996).

The antimicrobial activity of camel's milk and its whey protein fraction has already been studied (Benkerroum et al., 2004). Recently Salami and co-workers (2011) have studied the antimicrobial activity of camel whey proteins and their hydrolysates. However, to our knowledge, antimicrobial activity of camel caseins and their hydrolysates has never been studied. Search for biological activities of camel milk caseins has focused until now mainly on the antioxidant and angiotensin converting enzyme inhibitory activities (Salami et al., 2011).

Based on the above rationale, the objective of the present research is to assess whether hydrolysis by gastro-intestinal proteases of camel caseins releases antimicrobial fragments.

1. Materials and methods

1.1. Materials

Camel milk was obtained from the experimental farm of the Arid Land Institute, Livestock and Wildlife Laboratory, Tunisia. Pepsin (from porcine stomach mucosa, EC 3.24.3.1, specific activity of 3260 units mg^{-1}) and pancreatin (from bovine pancreas, EC 232-468-9, activity equivalent to 8× U.S.P. specifications), were from Sigma-Aldrich (Co., St. Louis, MO, USA).

1.2. Casein preparation

Caseins were extracted from skimmed camel milk by precipitation at pH 4.2 followed by centrifugation (5000×*g*; 30 min; 20 °C). The casein pellet was washed three times with distilled water, and then dissolved with 1 M NaOH. Then, caseins were dialyzed (cut-off of dialysis membranes: 100–500 Da, Spectra/Por, Spectrum Labs inc., Rancho Dominguez, CA, USA) against Milli-Q water (Millipore, Bedford, MA, USA) at 4 °C for 48 h and freeze-dried.

1.3. Casein enzymatic hydrolysis

Enzymatic hydrolysis protocol of camel milk casein was performed according to the conditions described by Parrot and co-workers (2003).

1.4. SDS PAGE electrophoresis

SDS-PolyAcrylamide Gel Electrophoresis (PAGE) was carried out according to method described by Laemmli and Favre (1973).

1.5. Nitrogen analysis by the Kjeldahl method

The levels of total nitrogen (TN) of CN were determined by the Kjeldahl method (AFNOR, 1993).

1.6. Gel filtration chromatography

The gel filtration chromatographic procedure was carried out using the same materials, methods, and condition as described by Dupas and co-workers (2009).

1.7. Peptide analysis by RP-HPLC

Peptide analysis was carried out by RP-HPLC/diode array detection, using the same protocol developed by ADT and co-workers (2011).

1.8. Antimicrobial activity assay

The in vitro antibacterial activity of CN and CN-H was assayed with a Bioscreen® automated spectrophotometer (Thermofisher, Illkirch, France) against 3 Gram-positive bacteria (*Listeria innocua* LRGIA01, *Bacillus cereus* ATCC 11778, and *Staphylococcus aureus* nosoco 3011) and 2 Gram-negative strains (*Escherichia coli* XL1 bleu and *Pseudomonas aeruginosa* ATCC 15742). The antimicrobial activity protocol is used according to JRAD and co-workers (2014).

1.9. Statistical analysis

Statistical analysis was performed using MS Excel and results were presented as mean value ± standard error of mean (SEM). The *t*-test (two-samples, assuming unequal variances) and P value <0.05 were used for statistical evaluation.

2. Results and discussion

2.1. Assessment of in vitro camel casein hydrolysis

In order to assess the in vitro hydrolysis of camel CN by gastrointestinal enzymes, the protein and peptide profiles were assayed by electrophoresis (Fig. 1). The SDS-PAGE pattern showed that undigested CN, which are considered as a heterogeneous group of milk proteins, co-migrate in the unique large band between 20 and 30 kDa. Therefore, the estimated molecular mass value of β-CN, κ-CN, α_{S1}-CN, and α_{S2}-CN in camel milk estimated by SDS-PAGE were 24.651 kDa, 18.254 kDa, 24.275 kDa, and 21.266 kDa, respectively (KAPPELER et al., 1998).

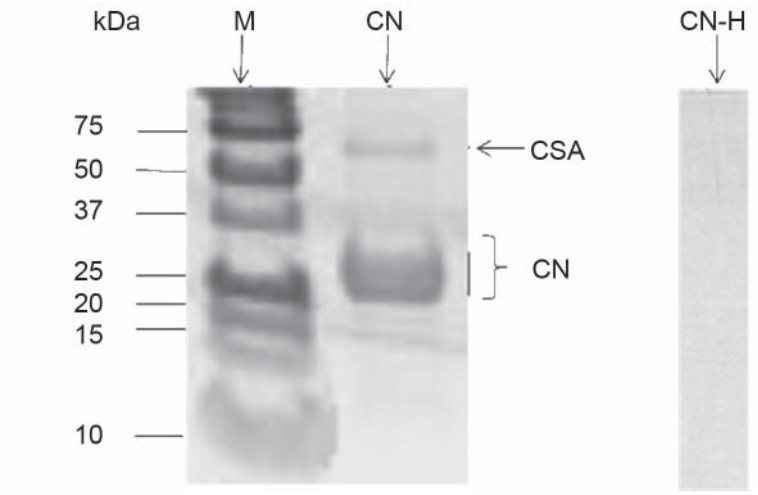

Fig. 1. SDS-PAGE electropherograms of CN and its enzymatic hydrolysate. M: molecular weight markers; CSA: camel serum albumin, CN: camel milk caseins

However, no more bands detectable by electrophoresis have been observed after a subsequent action of enzymes on CN, which were almost fully degraded by pepsin and pancreatin. This was consistent with non-protein nitrogen proportion in CN-H, which was 164 g l^{-1} (against 49 g l^{-1} in CN).

Before digestion, camel milk CN NPN is far lower than protein nitrogen. NPN/TN ratio was 11.5% in native CN, this indicates that only little peptides are present in camel CN. After hydrolysis, the (NPN/TN) ratio subsequently increased to 44%. This is consistent with PARROT and co-workers (2003) observation that (NPN/TN) ratio of bovine casein increased from 5 to 32% after hydrolysis by pepsin and pancreatin under the same conditions.

RP-HPLC chromatogram (Fig. 2) of CN sample revealed 2 major peaks eluted between 65 and 80 min., which were typical of large proteins like caseins, since the elution zone after 50 min is characteristic for large and/or hydrophobic peptides/proteins (PARROT et al., 2003). As a likely consequence of enzymatic hydrolysis, we can note the apparition of numerous peaks eluting between 20 and 60 min. These new peaks were the results of peptides liberation during the hydrolysis processes. This is consistent with the (NPN/TN) ratio increasing.

Peptides profiles were determined by gel filtration chromatography (GFC) and the molecular weight distribution was calculated using a calibration curve (Table 1).

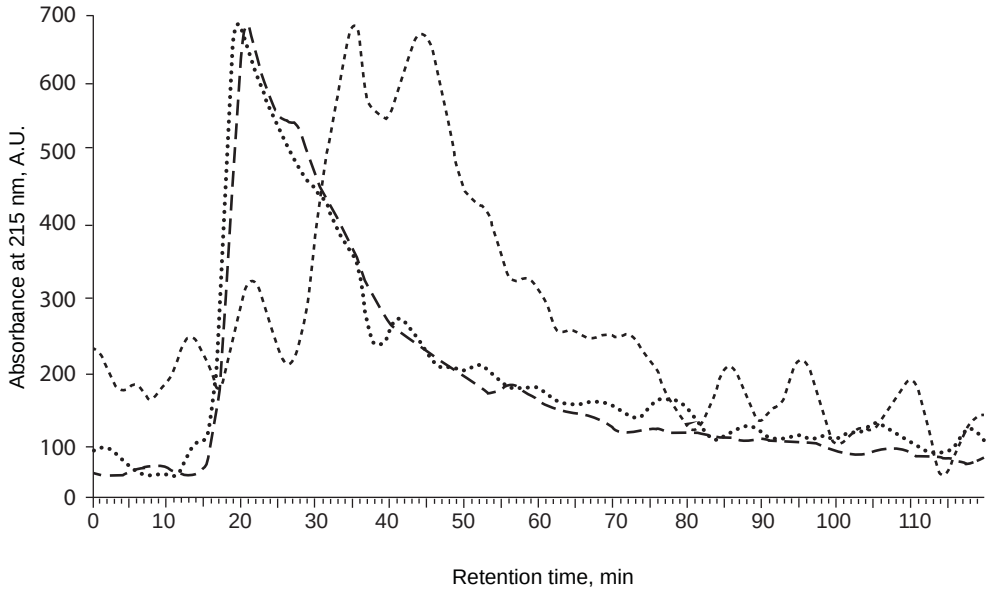

Fig. 2. Gel filtration chromatogram: separation of camel CN (• • • • •), its hydrolysate (CN-H - - - -), and 37 °C for 30 min and 4 h, respectively (in order to mimic successive conditions of hydrolysis by pepsin and pancreatin without addition of these enzymatic preparations) (CN: control – – –)

Table 1. Apparent molecular weight distribution of camel CN and CN-H by pepsin and pancreatin

Sample	Molecular weight distribution (%)			
	>10 kDa	5–10 kDa	1–5 kDa	<1 kDa
CN	24.2	10.3	27.2	38.3
CN-H	2.2	2.5	2.8	92.5

Apparent molecular weight distribution was calculated based on the respective areas of peaks of absorbance at 215 nm vs. elution time on the chromatograms obtained following analysis of CN and CN-H with a calibrated Superdex-Peptide® column as stated in the Materials and Methods section

The gel filtration chromatogram used for the determination of molecular weight distribution is presented in Fig. 3. Only one peak eluted with a retention time around 20 min was observed in the chromatogram of native CN, while following hydrolysis by gastro-intestinal proteases, several peaks with longer retention times were observed. These molecules, having a lower molecular mass than CN and an absorbance at 215 nm, likely correspond to caseins fragments released following their hydrolysis by digestive enzymes.

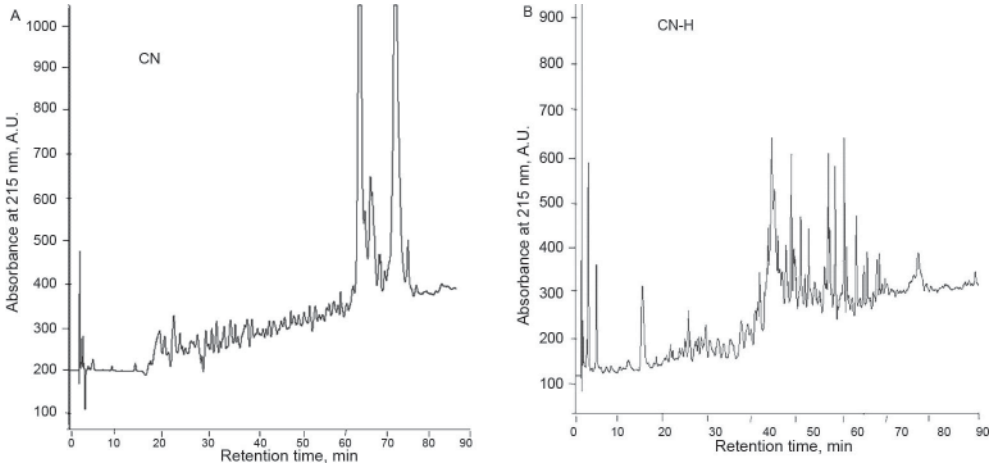

Fig. 3. RP-HPLC analysis of native camel CN (A) and their respective hydrolysate (B). See the Materials and Methods section for RP-HPLC conditions. A.U.: Absorbance Unit

After being subjected to the subsequent hydrolysis by pepsin and pancreatin, the proportion of molecules with an apparent molecular weight higher than 10 kDa decreased from 24.2 to 2.2%, while the proportion of molecules with an apparent molecular weight less than 1 kDa increased from 38.3 to 92.5%. A similar trend was observed with molecules with an apparent molecular weight between 5 and 10 kDa and 1 and 5 kDa: following enzymatic hydrolysis, their initial respective proportions (10.3 and 27.2%) decreased to 2.5 and 2.8%, respectively. These observations are consistent with the observation that CN band was no more visible on SDS-PAGE following their enzymatic hydrolysis.

2.2. Antibacterial activity assays

The antibacterial activity of camel CN before and after hydrolysis by gastro-intestinal enzymes against 3 Gram-positive bacteria (*L. innocua, B. cereus*, and *S. aureus* nosoco) and 2 Gram-negative bacteria (*E. coli* and *P. aeruginosa*) was assayed (Table 2). In the presence of 20 g l^{-1} of native camel CN, cell growth was significantly inhibited only for *E. coli* strain. The growth of the 4 other strains was either not significantly affected (*L. innocua*) or promoted (*B. cereus*, *S. aureus*, and *P. aeruginosa*). However, it can be noted that *L. innocua* growth was significantly inhibited in the presence of native CN at a 40 g l^{-1} concentration (Fig. 4). Interestingly, while camel CN hydrolysis by gastro-intestinal proteolytic enzymes likely released casein fragments, which could act as growth factors, the growth of the 5 bacterial strains tested was never promoted by camel CN-H at the same concentration when comparing with growth curves in the presence of native CN (Table 2). Except for *E. coli* growth, which was equally inhibited by native casein and its hydrolysate, it can be observed that the growth of all other bacterial strains was always slower in the presence of camel CN-H than in the presence of native camel milk CN. For instance, while *S. aureus* nosoco growth was slightly promoted by native camel CN, its growth was slightly inhibited in the presence of camel CN-H. Taken together, these observations suggest that fragments of camel CN inhibiting the growth of these bacterial strains were likely released by gastro-intestinal proteolytic enzymes. Since GFC analysis of molecular mass distribution indicates that the camel CN-H contained mainly peptides with an apparent molecular weight less than 1 kDa, it is likely that these antibacterial peptides also have a molecular weight lower than 1 kDa. This would be consistent with the observation of Silva and Malcata (2005) that antibacterial fragments derived from caseins have a molecular mass varying from 0.4 to 6 kDa.

Table 2. Inhibition/activation rate (%) of *L. innocua* LRGIA 01, *B. cereus* ATCC 11778, *S. aureus* nosoco 3011, *E. coli* XL1 blue, and *P. aeruginosa* ATCC 15742 growth by camel CN (at a concentration of 20 g l^{-1}) and its enzymatic hydrolysate (CN-H) after 15 h incubation at 30 °C

	Bacterial strain	CN	CN-H
Growth inhibition%	*L. innocua*	+2.55±0.14	−6.32±0.08
	P-value	P=0.83	P=0.42
	B. cereus	+11.61±0.02	–1.83±0.005
	P-value	P<0.001	P=0.8
	S. aureus nosoco	+8.98±0.07	–4.96±0.02
	P-value	P<0.001	P=0.21
	E. coli	–19.32±0.016	–19.73±0.01
	P-value	P<0.001	P<0.001
	P. aeruginosa	+14.83±0.026	+10.81±0.039
	P-value	P=0.026	P=0.0179

(+): stimulation of bacterial growth; (–): inhibition of bacterial growth

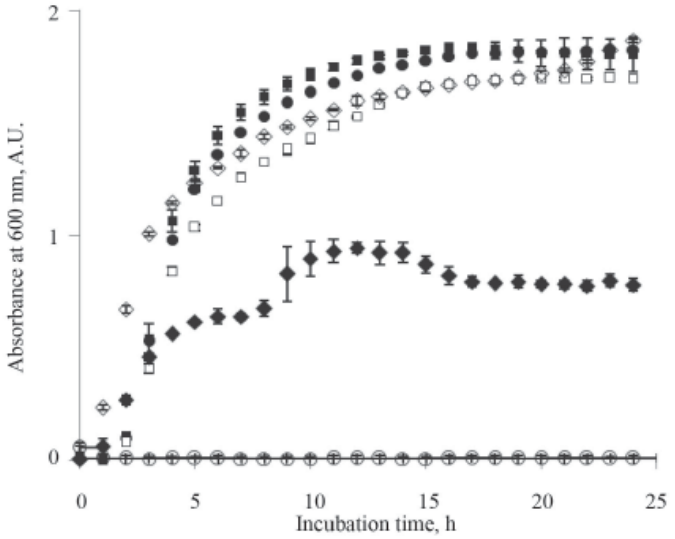

Fig. 4. Growth curves at 30 °C in BHI broth of *L. innocua* LRGIA01(•) in the presence of CN at a concentration of 20 g l^{-1} (■) and 40 g l^{-1} (♦), CN-H at a concentration of 20 g l^{-1} (□) and 10 g l^{-1} (◊) and nisin at 2400 IU ml^{-1} (○)

Hydrolysed CN are known to be highly complex mixtures that may contain up to hundreds of different peptides (Silva et al., 2006). In the present case, comparisons with other casein hydrolysates are further complicated due to differences in protein substrates and enzyme specificities of pepsin and the different proteolytic enzymes present in pancreatin. However, some similar trends with the hydrolysates of bovine and ovine caseins were observed: López-Expósito and co-workers (2006) reported that peptic hydrolysates of ovine α_{S2}-CN inhibited the growth of various Gram-positive bacteria.

Antimicrobial peptides derived from milk proteins often possess an amphiphilic and cationic character, which appears to be significant for their mechanism of action, since it is known that electrostatic bonding between the peptides and the bacterial membranes (negatively charged) is the initial stage of the pore formation process leading to cell death (Benkerroum, 2010).

3. Conclusions

In this study, camel caseins were hydrolyzed successively by pepsin and pancreatin mainly into fragments with a molecular weight below 1 kDa. The antibacterial activity of camel milk caseins was increased when compared with that of native casein after successive hydrolysis by proteolytic enzymes. This suggests that antibacterial fragments derived from camel caseins were released.

Future research should now be focused on the fractionation of camel CN digests by chromatographic procedures to purify and identify antibacterial peptides present in camel casein hydrolysates.

References

Adt, I., Dupas, C., Boutrou, R., Oulahal, N., Noel, C., Molle, D., Jouvet, T. & Degraeve, P. (2011): Identification of casein phosphopeptides generated through *in vitro* gastro-intestinal digestion of Beaufort cheese. *Int. Dairy J., 21,* 129–134.

AFNOR (1993): *Contrôle de la qualité des produits alimentaires: lait et produits laitiers: analyses physico-chimiques.* AFNOR (Ed.), Paris, France. 561 pages.

Benkerroum, N., Makkaoui, B., Bennani, N. & Kamal, H. (2004): Antimicrobial activity of camel's milk against pathogenic strains of *Escherichia coli* and *Listeria monocytogenes*. *Int. J. Dairy Technol., 57*, 39–43.

Benkerroum, N. (2010): Antimicrobial peptides generated from milk proteins: a survey and prospects for application in the food industry. A review. *Int. J. Dairy Technol., 63,* 320–338.

Dupas, C., Adt, I., Cottaz, A., Boutrou, R., Molle, D., Jardin, J., Jouvet, T. & Degraeve, P. (2009): A chromatographic procedure for semi-quantitative evaluation of casein phosphopeptides in cheese. *Dairy Sci. Technol., 89*, 519–529.

Jrad, Z., El Hatmi, H., Adt, I., Girardet, J.M., Cakir-Kiefer, C., Jardin, J., Degraeve, P., Khorchani, T. & Oulahal, N. (2014): Effect of digestive enzymes on antimicrobial, radical scavenging and angiotensin I-converting enzyme inhibitory activities of camel colostrum and milk proteins. *Dairy Sci. Technol., 94*, 205–224.

Kappeler, S., Farah, Z. & Puhan, Z. (1998): Sequence analysis of *Camelus dromedarius* milk caseins. *J. Dairy Res., 65*, 209–222.

Laemmli, U.K. & Favre, M. (1973): Maturation of the head of bacteriophage T4. I. DNA packaging events. *J. Mol. Biol., 80*, 575–579.

Lahov, E. & Regelson, W. (1996): Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. *Food. Chem. Toxicol., 34*, 131–145.

López-Expósito, I., Gómez-Ruiz, I.A., Amigo, L. & Recio, I. (2006): Identification of antibacterial peptides from ovine α_{S2}-CN. *Int. Dairy J., 16*, 1072–1080.

Parrot, S., Degraeve, P., Curia, C. & Martial-Gros, A. (2003): *In vitro* study on digestion of peptides in Emmental cheese: Analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. *Nahrung/ Food., 47,* 87–94.

Recio, I. & Visser, S. (1999): Identification of two distinct antibacterial domains within the sequence of bovine α s_2-casein. *Biochim. Biophys. Acta, 14*, 314–326.

Salami, M., Yousefi, R., Ehsani, M.R., Dalgalarrondo, M., Chobert, J.M., Haertlé, T., Razavi, S.H., Saboury, A.A., Niasari-Naslaji, A. & Moosavi-Movahedi A.A. (2008) Kinetic characterization of hydrolysis of camel and bovine milk proteins by pancreatic enzymes. *Int. Dairy J., 18*, 1097–1102.

Salami, M., Moosavi-Movahedi, A.A., Ehsani, M.R., Yousefi, R., Haertlé, T., Chobert, J.M., Razavi, S.H., Henrich, R., Balalaie, S., Ebadi, S.A., Pourtakdoost, S. & Niasari-Naslaji, A. (2010): Improvement of the antimicrobial and antioxidant activities of camel and bovine whey proteins by limited proteolysis. *J. Agric. Food Chem., 58*, 3297–3302.

Salami, M., Moosavi-Movahedi, A.A., Moosavi-Movahedi, F., Ehsani, M.R., Yousefi, R., Fahadi, M., Niasari-Naslaji, A., Saboury, A.A., Chobert, J.M. & Haertlé, T. (2011): Biological activity of camel milk casein following enzymatic digestion. *J. Dairy Res., 78*, 471–487.

Silva, S.V. & Malcata, F.X. (2005): Caseins as source of bioactive peptides. *Int. Dairy J., 15*, 1–15.

Silva, S.V., Pihlanto, A. & Malcata, F.X. (2006): Bioactive peptides in ovine and caprine cheese like systems prepared with proteases from *Cynara cardunculus*. *J. Dairy Sci., 89*, 3336–3344.

Zucht, H.D., Raida, M., Adermann, K., Magert, H.J. & Forssmann, W.G. (1995): Casocidin-I: A casein-as_2 derived peptide exhibits antibacterial activity. *FEBS Lett., 372*, 185–188.