Effect of pepsin-treated bovine and goat caseinomacropeptide on *Escherichia coli* and *Lactobacillus rhamnosus* in acidic conditions

G. Robitaille,¹ C. Lapointe, D. Leclerc, and M. Britten

Food Research and Development Centre (FRDC), Agriculture and Agri-Food Canada, St. Hyacinthe, Quebec, Canada, J2S 8E3

ABSTRACT

Caseinomacropeptide (CMP) is a 7-kDa phosphoglycopolypeptide released from κ -casein during milk digestion and in the cheesemaking process. The objective of the study was to analyze the effect of pepsin-treated CMP from cow and goat milk on the resistance of *Escherichia coli* and *Lactobacillus rhamnosus* during acid stress. Bacterial cells in the exponential growth phase were suspended in acidified phosphate buffered saline with or without pepsin-treated CMP. Viability was determined during a 90-min incubation period. Pepsin-treated CMP exhibited bactericidal activity at pH 3.5 when added in a dose-dependent manner to *E. coli*, decreasing survival by more than 90% within 15 min at 0.25 mg/mL. At pH >4.5, the bactericidal activity disappeared, indicating that pepsin-treated CMP was efficient at low pH only. The effectiveness of pepsin-treated CMP at pH 3.5 was not affected by the presence of glycoconjugates linked to CMP or by the bovine or caprine origin of milk. In contrast, *L. rhamnosus*, a probiotic, was more resistant to acid stress when pepsin-treated bovine or caprine CMP was added to the media. Viability reached 50% after 60 min of incubation at pH 3 compared with 5% survival in the media without added pepsin-treated CMP. Neither glycosylation extent nor sequence variations between CMP from bovine milk and caprine milk affected the protective activity of hydrolyzed CMP toward *L. rhamnosus*. This suggests that encrypted bioactive peptides released by the pepsin treatment of CMP had an antibacterial effect on *E. coli* in acidic media, but improved the resistance of *L. rhamnosus* to acid stress. The peptide fragment accountable for bactericidal activity is the N-terminal region κ -casein f(106–124).

Key words: caseinomacropeptide, acid stress, *Escherichia coli*, *Lactobacillus rhamnosus*

INTRODUCTION

Milk is a complete food, supplying nutrients for neonates as well as bioactive compounds, supporting the development and integrity of the intestinal mucosa and the establishment of adequate gut microflora, and providing natural defenses against pathogens. Besides the known antimicrobial compounds such as lysozyme, lactoperoxidase, and lactoferrin, there are antimicrobial peptides (AMP). Antimicrobial peptides are small amphiphilic peptides, usually cationic, which can integrate membrane bilayers to form pores that kill bacteria (Brogden, 2005). They are produced by the organism itself (e.g., α - and β -defensins), or are released from food-derived proteins by digestive enzymes (e.g., from milk proteins). Within the human gastrointestinal tract, AMP can assist innate immunity against pathogens along with gastric acidity, digestive enzymes, peristalsis, and the mucus layer. For neonates, who have an incomplete repertoire of intestinal microflora, protection by milk-derived AMP appears to be essential for healthy gut development, including intestinal epithelial homeostasis and mucosal healing (Bu et al., 2007). Because milk-derived AMP can be generally recognized as safe (GRAS), their use as a supplement in functional foods is an interesting alternative to the use of antibiotics (Benkerroum, 2010). Several milk-derived AMP have been reported from lactoferrin, α -LA, and β -LG, and from α_{S1} -, α_{S2} -, β -, and κ -CN (López-Expósito et al., 2008; Korhonen, 2009; Tidona et al., 2009; Benkerroum, 2010 for reviews).

Caseinomacropeptide (CMP) is a 7-kDa glycolphosphopolypeptide fragment produced by initial pepsin hydrolysis of κ -CN. It is also released from κ -CN into whey during chymosin-induced renneting of casein micelles in the cheesemaking process (Farrell et al., 2004). Bovine κ -CN is heterogeneous, presenting several molecular variations all located within the CMP portion of κ -CN, including genetic variants and post-translational glycovariants. The 2 most frequent genetic variants, A and B, are characterized by substitution $^{136}\text{Thr} \rightarrow ^{136}\text{Ile}$ and $^{148}\text{Asp} \rightarrow ^{148}\text{Ala}$. Up to 6 O-glycosylation sites and 5 different oligosaccharides having N-acetylneuraminic acid as terminal sugar, can be found. Bovine CMP is

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¹Corresponding author: robitaille@agr.gc.ca

a mixture of up to 14 glycovariants (**gCMP**) and a nonglycosylated form (**aCMP**); about 50% of bovine CMP are *O*-glycosylated (Mollé and Léonil, 2005). Caseinomacropeptide from goat milk differs significantly from bovine CMP by 19 AA substitutions and insertion of 2 AA, mainly located in the second half of the polypeptide. The extent of *O*-glycosylation of caprine CMP reaches only 30%, and *N*-glycolylneuraminic acid can also act as terminal sugar in addition to *N*-acetylneuraminic acid (Moreno et al., 2001). The vast majority of nonglycosylated isoforms of caprine CMP are diphosphorylated (Rasmussen et al., 1997), whereas bovine CMP is monophosphorylated. The differences between bovine CMP and caprine CMP can be highly informative when functional properties of CMP have to be analyzed.

Caseinomacropeptide is believed to have several local and systemic biological properties affecting the digestive, immune, cardiovascular, and nervous systems (Thomä-Worringer et al., 2006). It is reported that, when CMP is used as a food supplement, it can regulate microbiota and prevent infections (Brück et al., 2003, 2006a,b), decrease blood pressure (Miguel et al., 2007), and act on gastric hormones (Yvon et al., 1994). The *in vitro* approaches effectively indicate that CMP and CMP-derived peptides have immunostimulating (Li and Mine, 2004), antiadhesive (Neeser et al., 1988; Kawasaki et al., 2000), antihypertensive (Manso et al., 2002), antithrombotic (Jollès et al., 1986), opioid antagonism (Chiba et al., 1989), and antioxidative activities (López-Expósito et al., 2006). Furthermore, *in vitro* CMP and CMP-derived peptides have dual activity toward bacteria, depending on the species: growth-promoting activity in the case of *Bifidobacterium* genus (Azuma et al., 1984) and antimicrobial activity against several gram-positive and gram-negative species. For instance, Malkoski et al. (2001) reported antibacterial activity associated with kappacin, a specific isoform of bovine κ -CN peptide: the nonglycosylated phosphorylated CMP derived from κ -CN genetic variant A. The activity is centered within $^{149}\text{Ser(P)} \kappa\text{-CN-A } f(138\text{--}158)$, a peptide generated by an endoproteinase Glu-C. The bactericidal activity of kappacin varies greatly according to its molecular structure and integrity. As mentioned, only CMP from κ -CN-A is active; the B variant is completely inactive. This is an important consideration, given that the frequency of κ -CN-B is about 20%. Moreover, kappacin must be nonglycosylated, which restricts the active component to a specific isoform of CMP. Finally, it has been reported that bovine CMP is extensively cleaved by pepsin within the stomach (Chabance et al., 1995, Chabance et al., 1998); the active region $^{149}\text{Ser(P)} \kappa\text{-CN-A } f(138\text{--}158)$ is particularly sensitive, as it contains 5 proteolytic sites

(Mercier et al., 1972). Given all of these limitations, it is likely that the *in vivo* bactericidal activity kappacin is very low.

The acidic stomach environment is the first barrier against pathogens (Martinsen et al., 2005). The acidity of human stomach is dependent on food intake (type and amount) and on the time that has elapsed since the last meal. The luminal pH of around 2.0 (Dressman et al., 1990) can increase up to pH 5 after meal ingestion (Holzapfel et al., 1998). At pH 2, *Escherichia coli* is rapidly killed but at pH 3.5 the viability of *E. coli* can be higher than 50% even after 80 min of incubation (Zhu et al., 2006). Considering that gastric emptying is relatively fast, especially for liquids (Clarkston et al., 1997), the bactericidal efficiency of the stomach environment can be far from optimal. Incidentally, it is estimated that, on average, 20 to 80% of ingested *E. coli* can reach the small intestine without being inactivated by low pH (Takumi et al., 2000). Generation of food-derived AMP during gastric digestion could make a major contribution in terms of protecting against pathogens. On the other hand, the gastric environment can be detrimental to probiotics, bacterial strains which confer health benefits on the host when administered in adequate amounts (FAO/WHO, 2001). To be useful, food-derived AMP should be harmless for probiotics.

In all *in vitro* studies reporting milk-derived AMP activities, the antibacterial assays were conducted in optimal growth conditions, including neutral pH, in liquid culture media, or on medium-rich agar, by radial diffusion. In order for a milk-derived peptide to be recognized as an efficient AMP *in vivo*, validation in the gastrointestinal environment is required. The aim of the present study was to investigate the effect of the biological activity of pepsin-treated CMP on the survival of *E. coli*, including O157:H7, and on a probiotic, *Lactobacillus rhamnosus*, in acidic conditions, simulating a gastric environment. Pepsin-treated bovine CMP and caprine CMP were tested to evaluate the effects of glycosylation and AA sequence variations on the biological activity of CMP.

MATERIALS AND METHODS

Bacterial Strains and In Vitro Bactericidal Activity Test

Escherichia coli O157:H7 (ATCC 43888), *E. coli* DH5 α , and *L. rhamnosus* RW-9595 were obtained from the culture collection of the Food Research and Development Centre. *Escherichia coli* strains were cultured in brain heart infusion (BHI) broth at 37°C under aerobic conditions and *L. rhamnosus* was cultured anaerobically in de Man, Rogosa, and Sharpe

(MRS) broth at 37°C. To test bactericidal activity, an overnight bacterial culture was used to inoculate media at 1% (vol/vol). The culture was grown for 4 h at 37°C and centrifuged at $1,000 \times g$ for 5 min in a microcentrifuge. The bacterial pellet was washed in 0.85% NaCl (wt/vol), and suspended in PBS (pH 7.0) to an optical density of 0.1 at 600 nm. The test was carried out using a bacterial cell concentration of 10^5 to 10^6 cfu/mL for *E. coli* and 10^8 cfu/mL for *L. rhamnosus* at 37°C in PBS adjusted to the appropriate pH and containing 0.05 to 1 mg/mL of peptides. Before use, peptides were filter-sterilized (0.22- μ m filter unit; Millipore, Laval, QC, Canada). Samples were taken during incubation at 0, 30, 60, and 90 min, and viable bacterial counts (cfu/mL) were performed on Luria-Bertani broth (LB) agar for *E. coli* and MRS agar for *L. rhamnosus* using the pour plate technique. The plates were incubated at 37°C for 24 h under aerobic conditions for *E. coli* and anaerobic conditions for *L. rhamnosus*. The assays were conducted in triplicate and data from 3 independent replicate trials were analyzed using the GLM procedure (SAS Inst Inc., Cary, NC).

Peptide Preparation

Bovine milk from cows phenotyped κ -CN A was obtained from the Dairy and Swine Research and Development Centre (Agriculture and Agri-Food Canada, Sherbrooke, QC, Canada) and goat milk was from Laiterie Tournevent (Drummondville, QC, Canada). Phenotyping was carried out as described earlier (Robitaille et al., 1993). Milk was skimmed in a centrifugal separator (Elecrem3; Elecrem SA, Fresnes, France) at 35°C. Caseins were prepared by 2 cycles of acid precipitation at pH 4.6 with 5 N HCl and neutralization with 5 N NaOH. Finally, caseins were solubilized at 5% (wt/vol) at pH 6.3 and treated with chymosin (Maxiren 180, 180,000 milk clotting units/mL; Gist Brocades France SA, Seclin, France) at 37°C for 1 h. The reaction was stopped by means of acidification (pH 4.6) to precipitate paracasein. The supernatant containing CMP was concentrated and diafiltered by UF (molecular weight cut-off of 10 kDa), and lyophilized until use. Bovine CMP and caprine CMP were fractionated into aCMP and gCMP using anion-exchange membrane adsorption chromatography on a 60 mL Mustang Q cartridge [Pall (Canada) Ltd., Mississauga, ON, Canada]. Briefly, crude CMP was dissolved at 25 mg/mL in 25 mM Tris-HCl (pH 8.0) containing 6 M urea and absorbed on the column. The fraction aCMP and gCMP were desorbed using a linear 50 to 500 mM NaCl gradient in 25 mM Tris-HCl (pH 8) containing 6 M urea. Fractions were extensively dialyzed and lyophilized. The extent

of glycosylation, based on *N*-acetylneuraminic acid content, was evaluated spectrophotometrically by the acidic ninhydrin assay method (Fukuda et al., 2004). The concentration of CMP was determined using the extinction coefficient ($\epsilon_{1\text{cm } 214\text{ nm}}^{10\text{g/li}}$) of 140 (Coolbear et al., 1996).

To prepare pepsin-treated CMP, CMP was dissolved in deionized water at 5 mg/mL and the pH was adjusted to 2. Pepsin (EC 3.4.23.1, 3,460 U/mg; Sigma Chemical Co., St. Louis, MO) was added (1:100 enzyme:substrate ratio) and the mixture was incubated at 37°C for 3 h under agitation. The dispersion was heated to 80°C for 15 min to stop the reaction, neutralized to pH 7 by addition of 1 M NaOH, and centrifuged at $16,000 \times g$. The supernatant was recovered and lyophilized until use.

To purify the cationic peptide fraction, pepsin-treated CMP was solubilized at 10 mg/mL in 10 mM PBS (pH 7.0) at 10 mg/mL and applied on a Mustang S cartridge [Pall (Canada) Ltd., Mississauga, ON, Canada]. After extensive washing with the same buffer, the cationic peptide fraction was eluted with 10 mM phosphate (pH 7.0) containing 1 M NaCl. Cationic peptides were purified by reversed-phase HPLC (RP-HPLC) using a semi-preparative 250 $\times$ 10 mm column (Jupiter 10 μ m Proteo 90 A; Phenomenex Inc., Torrance, CA). The peptide was solubilized at 10 mg/mL in 0.1% trifluoroacetic acid (TFA) and 2 mL were injected and eluted using a 5 to 75% linear gradient of acetonitrile in 0.1% TFA at a flow rate of 3 mL/min. The fraction containing cationic peptides was collected, evaporated under nitrogen, and lyophilized. Synthetic κ -CN f(106–124) was synthesized and purified by GenScript USA Inc. (Piscataway, NJ). All chemicals were obtained from Fisher Scientific (Nepean, ON, Canada), unless otherwise specified.

For the spiking experiments, synthetic κ -CN f(106–124) was added to pepsin-treated CMP (0.2 mg/mg of pepsin-treated CMP) and analyzed by RP-HPLC on an ACE 5 C₁₈-300 column (150 $\times$ 4.6 mm; Canadian Life Science, Peterborough, ON, Canada) using a linear gradient of 30 to 50% (vol/vol) acetonitrile in 0.1% TFA at a flow rate of 1 mL/min at 20°C and at 50°C.

Sequence similarity between bovine CMP and caprine CMP was obtained using the MultAlin multiple sequence alignment program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSAHLN/npsahlp_alignmultalin.html). Peptide hydrophobicity was evaluated using the ProtScale program (<http://www.expasy.org/tools/protscale.html>). The net charge of the peptide over the entire pH range was estimated using Protein Calculator v3.3 (<http://www.scripps.edu/~cdputnam/protcalc.html>).

Figure 1. Survival of *Escherichia coli* DH5α during a 90-min incubation period in PBS with (A) or without (B) addition of 0.5 mg/mL of pepsin-treated bovine caseinomacropeptide (CMP). Incubation pH was 2.5 (× with vertical line), 3.0 (×), 3.5 (■), 4.0 (●), 4.5 (◆), and 7 (▲). Values ± standard errors are given for pH 3.5 and 4 only, to simplify presentation.

RESULTS AND DISCUSSION

The tolerance of *E. coli* DH5α to acid stress was assessed by evaluating the viability in PBS at pH 2.5 to pH 7 (Figure 1). At ≤pH 3, viability was decreased by more than 1.5 log₁₀ within 15 min, whereas it took 90 min of incubation at pH 3.5 to decrease the bacterial counts to that level. These results are similar to those reported by Zhu et al. (2006) showing that the viability of *E. coli* strains remains high after 80 min at pH 3.5. At pH >4, *E. coli* cell counts did not vary greatly ($P > 0.1$) for at least 90 min. This suggests a pH threshold between pH 3.0 and 3.5 at which *E. coli* becomes less sensitive to the bactericidal action of pH.

The addition of 0.5 mg/mL of pepsin-treated CMP to the incubation media induced significant bactericidal activity ($P < 0.05$), decreasing survival by about 0.7 log within 15 min and >2 log within 60 min (Figure 1). This bactericidal effect was detected only at a low pH level, as the bacteria were relatively resistant to the effect of pH ≥4.5. Moreover, intact bovine CMP (1 mg/mL) did not affect the viability of *E. coli* after 90 min of incubation at pH 3.5 (data not shown). These results indicate that, to express bactericidal activity,

Figure 2. Survival of *Escherichia coli* DH5α during a 90-min incubation period in PBS at pH 3.5. The concentration of added pepsin-treated caseinomacropeptide (CMP) was 0 mg/mL (×), 0.03 mg/mL (× with vertical line), 0.06 mg/mL (●), 0.12 mg/mL (▲), 0.25 mg/mL (◆), and 0.50 mg/mL (■).

encrypted peptides must be released by pepsin proteolysis of bovine CMP and that this bactericidal activity is observed only under acidic conditions.

To study the dose-dependent bactericidal activity at pH 3.5, various concentrations of pepsin-treated bovine CMP were added to the media (Figure 2). The bactericidal action of pepsin-treated bovine CMP showed an increasing trend up to 0.25 mg/mL. The pathogenic strain *E. coli* O157:H7 has been shown to be as sensitive as *E. coli* DH5α to pepsin-treated CMP at pH 3.5 (data not shown).

Glycoconjugates have been found to be implicated in many biological activities such as biological recognition processes, particularly with *N*-acetylneuraminic acid as the terminal sugar (Lehmann et al., 2006). To get some insight into the nature of the specific components or region of CMP that accounts for the bactericidal activity, pepsin-treated aCMP (<0.1 mol of neuraminic acid/mol) and gCMP (>3.5 moles of neuraminic acid/mol) originating from cow and goat milk were tested. As shown in Figure 3, pepsin-treated bovine and caprine aCMP at 0.5 mg/mL decreased the viability of *E. coli*, as did pepsin-treated bovine and caprine gCMP. This demonstrated that the bactericidal activity of pepsin-treated CMP was not related to the presence of oligosaccharide side chains.

A study was also conducted to evaluate the influence of pepsin-treated bovine CMP on *L. rhamnosus* viability. At pH 3.0, *L. rhamnosus* was sensitive to acid stress. The cell count decreased from about 1.5×10^8 cfu/mL to 1.5×10^6 cfu/mL after 60 min (Figure 4). When pepsin-treated bovine or caprine aCMP or gCMP were added at concentrations as low as 0.05 mg/mL, cell death was decreased by >1.2 log to reach 2.5

Figure 3. Effect of *O*-glycosylation on bactericidal activity of pepsin-treated caseinomacropeptide (CMP). Survival of *Escherichia coli* DH5 α during a 90-min incubation period in PBS (pH 3.5) without (x) or with added pepsin-treated nonglycosylated (■) or glycosylated (●) CMP (0.5 mg/mL) from bovine (solid line) and caprine (dashed line).

$\times 10^7$ cfu/mL. This demonstrated that pepsin-treated CMP can protect *L. rhamnosus* from acid stress and that its protective effect was not affected by the extent of glycosylation or the sequence variations due to the bovine and caprine origins of CMP.

The bactericidal activity of pepsin-treated CMP is dependent on AA sequence, as shown on Figure 3. The active peptide released by pepsin should be located within a region of homology between bovine and caprine CMP. Pepsin proteolysis of bovine CMP generates 14 peptides consisting of 2 to 19 AA (Mercier et al., 1972). The N-terminal peptide κ -CN f(106–124) is a good bactericidal candidate for several reasons. First, this region is well conserved between species (e.g., bovine, goat, human, common marmoset, pig, camel, and red deer) with identities $\geq 13/19$ AA, including a preserved sequence $^{107}\text{AIPPKKXQDK}^{116}$ and the 3 lysines at the same positions. Second, the isoelectric point is high, ranging from 8.25 to 9.7 for the majority of species. Furthermore, the hydrophobicity profile of bovine and caprine κ -CN f(106–124) is very similar, characterized by a cationic central core surrounded by 2 hydrophobic regions. Finally, κ -CN f(106–124) is proline rich, a characteristic shared by many cationic antibacterial peptides (<http://aps.unmc.edu/AP/main.html>). As mentioned by Shinnar et al. (2003), high proline content can improve resistance to digestion. Incidentally, it has been reported that κ -CN f(106–124) is rapidly generated within the stomach and is subsequently relatively resistant to gastric digestion (Chabance et al., 1995, 1998). This is for ensuring significant biological activity.

A synthetic peptide consisting of 19 AA encompassing κ -CN f(106–124) was synthesized and tested for bactericidal activity against *E. coli* DH5 α and *E.*

coli O157:H7 (ATCC 43888). The viability of *E. coli* O157:H7 decreased more rapidly at pH 3.5 in the presence of synthetic κ -CN f(106–124) compared with pepsin-treated CMP (Figure 2). The cell count decreased by more than 2 log₁₀ within 15 min at 0.5 mg/mL (Figure 5) and within 45 min at 0.05 mg/mL (data not shown). Moreover, the bactericidal activity showed pH dependency similar to the pepsin-treated bovine CMP. The resistance of *E. coli* O157:H7 was relatively high at pH 4.0 compared with pH 3.5, decreasing by only 0.2 log after 30 min; after that, it continued to gradually decrease, eventually reaching 1.1 log at 90 min. At pH 4.5, *E. coli* was resistant to acid stress, with or without synthetic κ -CN f(106–124). The loss of activity observed in the presence of the synthetic κ -casein f(106–124) peptide at higher pH is probably due to the decrease in the cationic character of the peptide. In fact, it is estimated that an increase in pH from 3.5 to 6.5 decreases the net charge of κ -CN f(106–124) from +3.1 to +1 due to deprotonation of ^{114}Asp in particular. Considering that the initial step of the proposed mechanism of action of cationic AMP is electrostatic interaction between the cationic segment of AMP and negatively charged phospholipid head groups (Brogden, 2005), the loss of positive charges would weaken these interactions.

Reversed-phase HPLC analysis of κ -CN f(106–124) and pepsin-treated bovine CMP was conducted (Figure 6 and 7). At 20°C, the synthetic κ -CN f(106–124) was resolved into 2 peaks at 27.8 and 31.6 min on an HPLC C₁₈ column (Figure 6a). These 2 peaks were also found in the cationic CMP purified by cation-exchange chromatography followed by preparative RP-HPLC (Figure 6d). The same 2 peaks increased in relative intensity in

Figure 4. Effect of added pepsin-treated caseinomacropeptide (CMP) on survival of *Lactobacillus rhamnosus* RW-9595M during a 90-min incubation period in PBS (pH 3) without (x) and with added pepsin-treated nonglycosylated (■) or glycosylated (●) bovine (solid line) and caprine (dashed line) CMP (0.5 mg/mL).

Figure 5. Survival of *Escherichia coli* O157:H7 during a 90-min incubation period in PBS at pH 3.5 (■) or at pH 4.0 (●) with addition of 0.5 mg/mL of synthetic κ -CN f(106–124). Control at pH 3.5 without added peptide (◆).

the spiked sample (Figure 6c) containing pepsin-treated CMP and synthetic κ -CN f(106–124).

The chromatogram from the RP-HPLC analysis of synthetic κ -CN f(106–124) at 50°C showed that the peptide was eluted as a broad peak with a retention time centered on 24.7 min (Figure 7a). A similar peak shape was detected at the position of pepsin-treated CMP (Figure 7b) in the chromatogram and in the spiked sample (Figure 7c), where it specifically increased in intensity. The elution of synthetic κ -CN f(106–124) as a doublet at 20°C and as a broad band at 50°C can be explained by the presence of prolines in tandem. It has been shown that linear peptides with a proline-proline bond show a slow conformational equilibrium that induces peak broadening or peak splitting, depending on the temperature during RP-HPLC separation (Gesquiere et al., 1989). The similarities between the

Figure 6. Reversed-phase HPLC elution profile of (a) synthetic κ -CN f(106–124), (b) pepsin-treated caseinomacropeptide (CMP), (c) spiked sample, and (d) cationic CMP peptide. Linear gradient of 30 to 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) on a C₁₈ column at 20°C.

Figure 7. Reversed-phase HPLC elution profile of (a) synthetic κ -CN f(106–124), (b) pepsin-treated caseinomacropeptide (CMP), and (c) a spiked sample. Linear gradient of 30 to 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) on a C₁₈ column at 50°C.

RP-HPLC profiles of synthetic κ -CN f(106–124) and the cationic CMP and its detection in pepsin-treated CMP corroborate the hypothesis that the bactericidal peptide κ -CN f(106–124) is generated by pepsin hydrolysis of CMP in acidic conditions.

Besides exhibiting bactericidal activity against *E. coli* as demonstrated in this study, κ -casein f(106–124) includes the fragment κ -CN f(106–116), which is detected in the duodenum for several hours and then absorbed by the intestinal mucosa (Chabance et al. 1998). κ -Casein f(106–116) is antithrombotic, inhibiting ADP-induced platelet aggregation and the binding of fibrinogen to platelets (Jollès et al., 1986). This type of multifunctional role is observed with other AMP, such as lactoferricin from pepsin-treated lactoferrin, which has antimicrobial, antifungal, antitumoral, immunoregulatory (Wakabayashi et al., 2006), and anti-hypertensive activities (Ruiz-Giménez et al., 2010).

In conclusion, CMP is the first casein fragment produced from κ -CN during gastric digestion. Caseinomacropeptide is rapidly hydrolyzed into shorter peptides under the action of pepsin. One of these peptides, the positively charged peptide κ -CN f(106–124), can act as an antibacterial agent against *E. coli* during the gastric phase. A systematic analysis of gram-positive and gram-negative bacteria is being carried out to evaluate the efficiency of κ -CN f(106–124). Preliminary results indicate that the peptide is also active against gram-positive *Listeria monocytogenes*.

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