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Glycosylations of κ-Casein-Derived Caseinomacropeptide Reduce Its Accessibility to Endo- but Not Exointestinal Brush Border Membrane Peptidases

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Caseinomacropeptide (CMP) is a peptide obtained from κ-casein hydrolysis by gastric proteinases and which exhibits various biological activities. The aim of this study was to analyze the intestinal processing of CMP at the brush border membrane (BBM) level. Intestinal BBM vesicles (BBMV) were used to digest glycosylated and unglycosylated CMP. Our results demonstrated that whatever was the glycosylated state of CMP, they were digested by BBMV intestinal enzymes, from macropeptides to free amino acids. The digestion of unglycosylated and glycosylated CMP throughout the action of exopeptidases was similar, but the activity of endopeptideases on glycosylated CMP was limited, certainly due to the attached *O*-glycosylations. Consequently, much more peptides were identified from the unglycosylated than from the glycosylated CMP. In addition, the glycosylation core as well as the number of the attached glycosylated chain modified the kinetic of digestion; the most heavily glycosylated forms being the slowest digested.

KEYWORDS: Caseinomacropeptide; glycosylation; intestinal digestion; brush border membrane vesicles

INTRODUCTION

Caseinomacropeptide (CMP) is a 64 amino acid residue peptide obtained from κ-casein (κ-CN) hydrolysis during the first phase of the digestion by gastric proteinases, which rapidly hydrolyzes the $Phe_{105}-Met_{106}$ bond, producing a N-terminal fragment (para-κ-CN, residue 1−105) and CMP (residue 106−169). CMP is frequently referred to as glycomacropeptide (GMP) that is a heterogeneous group of peptides having the same peptide chain but variable glycoside and phosphorus content. CMP only possess O-glycosidic linkages, the number of glycosylation sites varying from 0 to 7 (*1, 2*). Thus, the molecular mass of the different molecular forms of GMP varies from 6755 up to 9626 Da (*1, 2*). CMP released from κ-CN is a predominant breakdown product of casein in the human stomach (*3, 4*). It is released in the human jejunum following whey protein, casein, and yogurt ingestion (*3, 5*). It has even been shown that CMP crosses the intestinal epithelium because it is found in the blood after milk or yogurt ingestion (*5*).

The release and subsequent absorption of CMP during milk protein digestion in the intestine could be of nutritional and physiological significance since CMP or its fragments have been shown to have various biological activities as physiological regulators, either directly as neurotransmitters or indirectly by modulating secretion of intestinal hormones (*6*−*10*). In the intestine, CMP has an inhibitory effect on gastric acid secretions (*11*) and is able to stimulate pancreatic secretion through the release of cholecystokinin (*12, 13*), although no effect could be demonstrated on satiety (*14, 15*). CMP through its antithrombotic features (*16*) and antihypertensive activity (*17*) can modulate the cardiovascular system. It has also been shown that CMP can exert immunomodulating effects and antimicrobial activities by inhibiting a pathogen's adhesion to cells (*18, 19*), as well as influenza viruses (*20, 21*). To express these biological activities in vivo, CMP or smaller derived peptides have to reach their target.

An important step in the intestinal processing of food protein-derived peptides takes place at the brush border membrane (BBM) level. This membrane, which covers the entire surface of the small intestine, contains many hydrolytic enzymes and transport systems involved in final digestion and nutrient absorption. The BBM expresses at least 15 peptidases that are involved in the terminal stages of protein digestion (*22*); they have a broad specificity and can digest both proteins and peptides. Various classes of peptidases have been detected in

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human intestinal BBM (*23*): endopeptidases, aminopeptidases, and carboxypeptidases and the dipeptidase IV. The brush border peptidases face outward from the membrane into the intestinal lumen, to hydrolyze peptides that come into contact with the cell surface.

The aim of the present study was to describe the digestion profile of glycosylated and unglycosylated CMP throughout the action of the small intestinal brush border enzymes. Our original digestion model used pig brush border membrane vesicles (BBMV) to hydrolyze CMP. We exploited the advantage of this model to present all of the intestinal enzymes involved in the digestion of nutrients although BBMV are usually used to isolate and characterize intestinal BBM enzymes and to study intestinal transport, uptake, or absorption. The breakdown of both substrates and their kinetics of digestion was evaluated, and the peptides released from them were identified.

MATERIALS AND METHODS

Preparation of CMP0 and GMPs. This preparation was achieved once with about 14 L of fresh whole milk from cows homozygous for the κ-CN A variant. Milk was skimmed in a centrifugal separator (Elecrem3, France) at 35 °C, and whole casein was prepared by isoelectric precipitation at pH 4.6 using 1 M HCl. The casein precipitate was washed three times with water. For the preparation of sodium caseinate, the precipitate was dissolved in distilled water adjusted to pH 6.3 with 1 M NaOH. CMP was prepared by the action of chymosin (Maxiren 180, 180000 milk clotting units/mL; Gist-brocades, Seclin, France). Briefly, sodium caseinate solution was hydrolyzed for 40 min at pH 6.3 and 35 °C at an enzyme/substrate ratio of 1:180000 (w:v). The reaction was stopped by adding 1 M HCl until pH 4.6 to precipitate the nonhydrolyzed casein fraction. The CMP supernatant was extracted using 2% trichloroacetic acid (TCA; Sigma, Saint-Quentin Fallavier, France). Unglycosylated CMP (CMP0) and glycosylated CMP (GMPs) were separated using a final concentration of 12% TCA. After centrifugation (5000*g*, 10 min), the pellet that contained the CMP0 was dissolved in distilled water, the pH was adjusted to 6.7 with 1 M NaOH, and the solution was dialyzed through a 3500 Da cutoff membrane tubing (Spectra/Por, Medicell International Ltd., London, United Kingdom) for 72 h at 4 °C against distilled water. The 12% TCA-treated supernatant, which contained GMPs, was similarly dialyzed. Both dialyzed preparations were lyophilized and stored at 4 °C.

Preparation of BBMV. The jejunum of a freshly killed pig (male weighing 50 kg) was removed and rinsed with cold 0.9% NaCl. All subsequent steps were performed on ice or at 4 °C. The intestinal mucosa was scraped off with a glass slide and homogenized in a Warring blender (Grosseron, Saint Herblain, France) at full speed twice for 30 s in 20 vol (w:v) of homogenate media (50 mM mannitol in 2 mM Tris-HCl buffer, pH 7.0, and 0.1 mM PMSF). Calcium chloride was then added to a final concentration of 12.5 mM, and the suspension was stirred in an ice bath for 1 h. The suspension was centrifuged (5000*g*, 15 min at 4 °C). The pellet was discarded, and the supernatant was subjected to a second centrifugation at 12000*g* for 30 min at 4 °C. The resulting pellet containing the crude brush border fragments was disrupted into microvillus membrane in homogenate media (1 mL for 4 g of mucosa) using a 2 mL syringe with a 0.5 mm × 16 mm needle. A 0.5 mL amount of sample was frozen and stored in liquid N_2 until use. Purification and enrichment of the BBMV were checked by determination of the marker enzymes: alkaline phosphatase (EC 3.1.3.1) and dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.5). To measure the alkaline phosphatase activity, samples were diluted 1:100 in 0.1 M sodium carbonate buffer, pH 9.4, and mixed to an equal volume of *para*-nitrophenyl phosphate (Sigma). The absorbance at 405 nm was measured each minute for 10 min to determine the activity. To measure the DPPIV activity, samples were diluted 1:80 in 0.02 M Tris-HCl buffer, pH 7.5. Fifty microliters was incubated with 50 μL of 0.66 mM Phe-Pro β-naphtylamide (Bachem, Weil am Rhein, Germany) at 37 °C. The reaction was stopped by adding 50 μL of a mixture containing 1 mg/mL Fast Garnet (Sigma), 10% (v:v) Triton X-100, and 1 M sodium acetate, pH 4.0, after 0, 5, 10, 15, and 20 min, and the absorbance at 550 nm was measured. The protein concentration was determined by using the Bradford reagent (Sigma) with bovine serum albumin as a standard. The specific alkaline phosphate and DPPIV activities were 19.7- and 22.9-fold enriched, respectively, in the final BBMV fraction. In this preparation, the contribution of microbial enzymes was excluded as far as BBMV were free of microorganisms. Indeed, bacteria were first eliminated from mucosa during the abundant rinsing of the intestine before the scraping; thereafter, they burst under osmotic pressure and/or were eliminated under the second centrifugation.

Assessment of CMP0 and GMPs Digestion by BBMV. Digestion of CMP0 and GMPs was performed at 37 °C in 35 mM Hepes-Tris buffer and 0.15 M KCl, pH 7.0. The VMBB/substrate ratio was evaluated in a preliminary study to be able to monitor the digestion kinetics. Digestion was started by mixing an equal volume of the substrate solution (5 g/L) and BBMV preparation diluted 1:5 in Hepes-Tris buffer. At selected times, 0.3 mL samples were collected, and the reaction was stopped by centrifugating BBMV (2000*g* for 30 s). The supernatant was stored at −20 °C until analysis. A blank sample was realized by adding the buffer without CMP, and for control, CMPs were incubated without BBMV.

Global digestion was determined by measuring free amino groups with trinitrobenzenesulfonic acid (Sigma) as previously described (*24*) after 1:2 dilution in distilled water. Free amino acids (FAAs) produced throughout digestion were determined as previously described (*25*) after precipitation of peptides with 3% sulfosalicylic acid. The concentration of each of the 20 FAAs analyzed was summed to estimate the total amount of FAAs.

Identification of Peptides by Nano-LC/MS-MS. All mass spectra were performed using a hybrid quadrupole time-of-flight (Q/TOF) mass spectrometer QStar XL (MDS Sciex, Toronto, Canada). The instrument was calibrated with a multipoint calibration using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein, β-CN (193−209) (NeoMPS S.A., Strasbourg, France). After 1:25 dilution in 0.1% trifluoroacetic acid (TFA; Pierce, Touzart et Matignon, Vitry-sur-Seine, France), the peptide fraction (10 μL) was trapped onto a micropre-column cartridge C_{18} PepMap 100 (300 μm i.d.−5 mm, Dionex) before separation of peptides onto a column C_{18} PepMap (75 μm i.d.−150 mm, Dionex). The separation started with 10% solvent B for 5 min, and a linear gradient from 10 to 50% solvent B for 45 min was performed at a flow rate of 200 nL/min. Solvent A contained 2% acetonitrile, 0.08% formic acid, and 0.01% TFA in LC grade water, and solvent B contained 95% acetonitrile, 0.08% formic acid, and 0.01% TFA in LC grade water.

The online separated peptides were analyzed by ESI Q-TOF in positive ion mode. A voltage of around 3.8 and 3.6 kV for CMP0 and GMPs, respectively, was applied to the nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). MS and MS/MS data were acquired in continuum mode. Data-direct analysis was employed to perform MS/MS analysis on 1+ to 3+ charged precursor ions. Precursor selection was based upon ion intensity and charge state, and if the precursors had been previously selected for interrogation, they were excluded for the rest of the analysis. Spectra were collected in the selected mass range 350−2500 *m/z* for MS and 60−2500 *m/z* for MS/MS spectra. The mass spectrometer was operated in data-dependent mode automatically switching between MS and MS/MS acquisition using Analyst QS 1.1 software (Applied Biosystems, Framingham, MA) when the intensity of the ions was above 5 cps.

To identify peptides, all data (MS and MS/MS) were submitted to MASCOT (v.2.2). The search was performed against a homemade database dealing with major milk proteins, which represents a portion of the Swissprot database (http://www.expasy.org). No specific enzyme cleavage was used, and the peptide mass tolerance was set to 0.8 Da for MS and 0.3 Da for MS/MS. Eight variable modifications dealing with glycosylation on serine and threonine residues were selected. For each peptide identified, a minimum MASCOT score of at least 20 was considered as a prerequisite for peptide validation with a high degree of confidence.

Identification and quantification of glycosylated and phosphorylated macropeptides were performed using the Analyst QS 1.1 software. They

Table 1. Data Sheet of Individual GMPs and Phosphorylated CMP Identified in Our Total GMPs Preparation[a]

	observed molecular mass (Da)	calculated molecular mass (Da)	retention time (min)
CMP 1P	6787.88	6787.42	45.6
CMP 2P	6867.35	6867.33	46.8
CMP 1C/D[b]	7443.95	7444.10	45.4
CMP 1E	7735.18	7735.27	47.0
CMP 2C/D[b] ou 1E 1B	8100.71	8100.60	41.7
CMP 1E 1C/D[b]	8391.86	8391.85	43.3
CMP 2E	8683.29	8683.10	46.1
CMP 1E 2C/D[b] ou 2E 1B	9048.50	9048.42	45.6

[a] The structure of the carbohydrate chains linked to GMPs is also described in the scheme where Gal is galactose, NeuAc is *N*-acetylneuraminic acid, and GalNAc is *N*-acetylgalactosamine. Their molecular masses are as follows: B = 383.3 Da, C = 674.6 Da, D = 674.6 Da, and E = 965.8 Da. (B) Galβ (1−3) GalNAc, (C) NeuAcα (2−3) Galβ (1−3) GalNAc NeuAc l2−6α, (D) Galβ (l−3) GalNAc NeuAc l2−6α, and (E) NeuAcα (2−3) Galβ (l−3) GalNAc. [b] C and D (C/D), having the same mass, cannot be discriminated by MS analysis.

were identified in MS according to their molecular mass as determined by Mollé and Léonil (*2*) and presented in **Table 1**. After extraction of the MS scans in the region where GMPs eluted, the "Bayesian Peptide Reconstruct" tool was used to calculate the macropeptides masses and their intensities from the *m*/*z* and intensity values of the multiply charged ions. The same number of MS scans was used for all samples to ensure comparable intensity results.

RESULTS

Assessment of Digestion. The measure of free amino groups, that is, the number of peptide bonds cleaved by endopeptidases and the nonaromatic amino acids released through the action of exopeptidases, allowed evaluation of global digestion state. From either CMP0 or GMPs, the quantity of free amino groups largely increased during the first 8 h of digestion (**Figure 1A**). This increase showed the significant proteolytic activity of BBMV. The digestion time course, which was linear up to 8 h, was twice higher for CMP0 than for GMPs (see slopes in **Figure 1A**). Thereafter, the quantity of free amino groups decreased a little for CMP0 and GMPs digestion. The analysis of a blank sample, that is, BBMV without CMP, allowed us to estimate that the amino groups that could arise from digestion of endo- and exopeptidases was negligible, 0.43 ± 0.14 and 0.60 ± 0.26 mM at 0 and 24 h, respectively.

FAAs were continuously released through the action of exopeptidases, that is, carboxy- and aminopeptidases, during the first 8 h of digestion (**Figure 1B**). Moreover, the release time course was linear and similar for CMP0 and GMPs until 3 h of digestion (see slope, **Figure 1B**). It remained unchanged until 8 for CMP0, whereas it decreased for GMPs. As for free amino groups, the FAAs concentration decreased from 8 to 24 h of digestion for both substrates. A blank of BBMV allowed us to estimate that the FAAs that could arise from digestion of endo- and exopeptidases was negligible, 0.11 ± 0.11 and 0.06 ± 0.03 mM at 0 and 24 h, respectively. The concentration of FAAs decreased from 8 to 24 h of digestion; they were hydrolyzed into NH_3, whose quantity increased from 8 to 24 h of digestion (data not shown).

Kinetics of the Digestion. The disappearance of each substrate was evaluated using peak intensity as determined by mass spectrometry (MS). The intensity of CMP0 decreased linearly during the first 3 h of digestion, the time needed to digest almost all of the substrate (**Figure 2A**). The intensity of total GMPs was calculated as the sum of each glycosylated form detected within GMPs preparation using mass analysis by MS (**Table 1**). The intensity of total GMPs decreased exponentially.

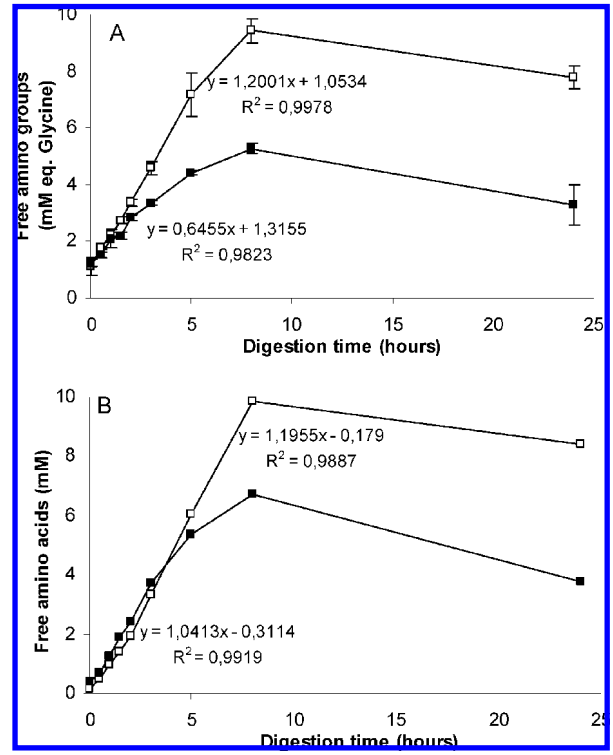

Figure 1. Determination of free amino groups (**A**) and FAAs (**B**) in the hydrolysates from CMP0 (□) and total GMPs (■) during the digestion by enzymes from BBMV. The equations of appearance of free amino groups and FAAS have been calculated considering the first 5 h of digestion.

Thus, a plot of CMP0 intensity vs digestion time allowed us to determine the half-life $t_{1/2}$, that is, the time required for the disappearance of one-half of substrate under digestion; however, the log of GMPs intensity vs digestion time was plotted to determine the GMPs half-life $t_{1/2}$ (**Figure 2**B). Half-lives values of 1.8 and 3.75 h were, respectively, determined for CMP0 and GMPs.

Among the five oligosaccharides reported for GMP (*26*), the glycosylation cores B, C, or D and E have been identified in our GMPs preparation (see **Table 1**). Because the initial intensity of the identified GMPs varied from one to each other, the intensity percentage was used to compare their kinetics of digestion. In relation to the kinetic of total GMPs digestion, the results showed that the intensity of each glycosylated form decreased exponentially (**Figure 3**). All of the glycosylated substrates were almost digested within 5 h whatever was the glycosylation core. Considering the glycosylation core, we observed that the GMP-trisaccharide C or D was faster digested than the GMP-tetrasaccharide E, which was also digested more rapidly than the forms having two oligosaccharides. These results showed that as the oligosaccharide chain got heavier and as the glycosylation rate increased, the substrate digestion was slowed down. Phosphorylations present on CMP, ranging from one to two in our experiment, were at least as rapidly released as the smallest glycosylation core (**Figure 3**).

Identification of Peptides. All of the samples were analyzed by nano-ESI-MS/MS to identify the peptides released throughout the digestion. Only the results from samples obtained after 0.5, 3, and 8 h of digestion are presented (**Figure 4**). The total ion current (TIC) profiles allowed visualization of the substrate breakdown during the digestion. CMP0 (peak between 46 and 47.5 min) decreased up to and disappeared after 5 h of digestion. The number of digestion products increased throughout the first hours of digestion (peptides eluted from 32−36 and 39−43

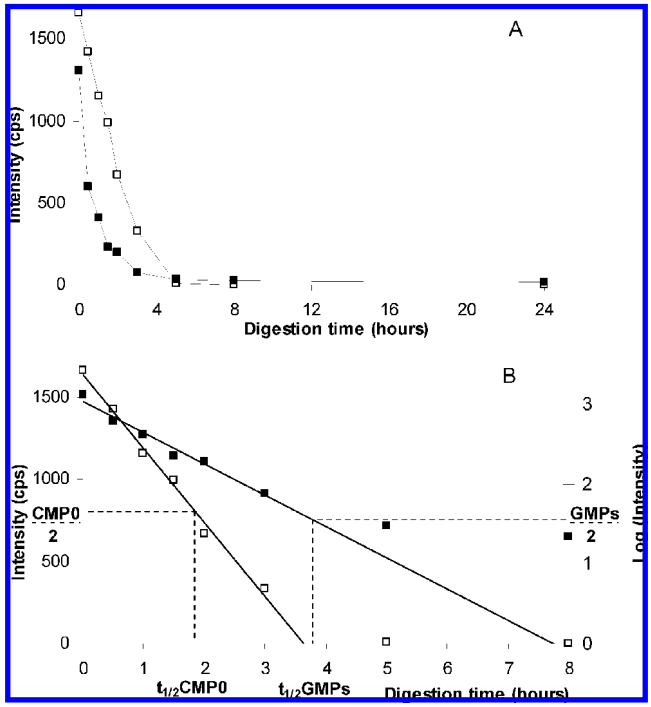

Figure 2. Digestion kinetics showed the order of the reaction (**A**) and were used to determine the half-life $t_{1/2}$ (**B**) for CMP0 (□) and total GMPs (■).

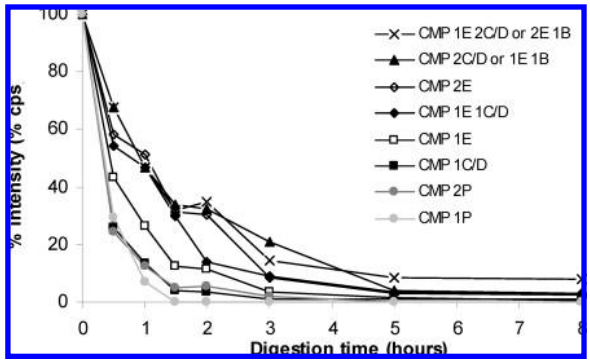

Figure 3. Disappearance of each glycosylated form and the phosphorylated forms of the GMP, presented as a percentage of the relative intensity of each glycosylated form identified, and normalized to 100% at the beginning of the digestion.

min), and these products were themselves further hydrolyzed (peptides eluted from 18 to 32 min) (**Figure 4A**). All of the different glycosylated forms of GMPs were eluted from 41.5 to 47 min (**Figure 4B**). The digestion pattern of GMPs differed from that of CMP0. The major peak that remained after 8 and 24 h of GMPs digestion was eluted from 36 to 41 min.

Many more peptides were identified from CMP0 than from GMPs digestion (**Tables 2** and **3**). For example, after 3 h of digestion, 66 and 13 peptides were identified for CMP0 and GMPs, respectively. For both substrates, the C-terminal end of the sequence was first degraded, namely, the sequence 148−169 in our study. Diversity of the peptides identified was the highest after 2 or 3 h of GMPs or CMP0 digestion. Thereafter, the number of peptides obtained from the C-terminal end of the sequence decreased, and after 24 h of digestion, only one or two peptides were detected. An in-depth analysis of the identified peptides allowed us to assume the cleavage sites of the endopeptidases (**Tables 2** and **3**). For example, the bond Glu_{147}−Asp_{148} might be one of the first cleaved. Subsequently, the C-terminal end of the sequence was degraded through the action of both amino- and carboxypeptidases. The N-terminal end of the sequence, that is, the sequence 106−148, was digested later than the C-terminal one. After 8 h of digestion, most of the identified peptides originated from the N-terminal end.

Most of the glycosylation sites are located in the middle part of the sequence. However, two of the three glycosylated peptides identified from the digested GMPs originated from the C-terminal end of the GMPs where only one glycosylation is attached. These glycosylated peptides, the κ-CN (156−168) and κ-CN (155−169), were identified rapidly after 0.5 h of digestion up to 24 h (**Table 3**). Deduced from their masses, they had a C/D and an E glycosylation, respectively. In addition to these two peptides, the glycosylated peptide κ-CN (129−146) was identified after 8−24 h of digestion. Surprisingly, it is the only glycosylated peptide obtained from the most glycosylated part of the GMPs sequence. According to its mass, the peptide κ-CN (129−146) supported an E glycosylation.

Interestingly, the peptide κ-CN (115−121) was identified in the 24 h hydrolysate obtained from both CMP0 and GMPs. Its intensity increased exponentially up to 8 h of digestion and remained almost stable until 24 h (**Figure 5**). In similar initial substrate concentration, this peptide was much more released from CMP0 than from GMPs; its maximum intensity was 4.5 times higher for CMP0 than for GMPs. Another peptide, the κ-CN (155−161), was also identified until 8 h of digestion in both hydrolysates, but it almost disappeared at 24 h (data not shown). Its maximum intensity was 1.3 times higher for CMP0 than for GMPs.

DISCUSSION

In the present study, pig BBMVs were used to evaluate the intestinal BBM digestion of CMP, a peptide endowed with numerous biological activities, in both its unglycosylated (CMP0) and its various glycosylated forms (GMPs). Although it is well-established that CMP is the initial and predominant breakdown product of κ-CN in the human stomach and that it is thereafter digested by pancreatic enzymes (*27, 28*), when it is released in the jejunum of humans (*29*), a part of the nonhydrolyzed CMP reaches the BBM where it may be absorbed as suggested by its retrieval in the blood (*29, 30*). Our results show for the first time that CMP0 and GMPs can be hydrolyzed in the intestinal lumen by the BBM enzymes.

The release time course of both free amino groups and FAAs throughout the digestion of CMP0 and GMPs allowed comparison of the endopeptidases activity to the exopeptidases one (**Figure 1**). Indeed, the large exopepdidase activity mainly contributed to the release of free amino groups. Insofar as the digestion conditions for CMP0 and GMPs were identical, that is, the same substrate concentration, BBMV concentration, and substrate/BBMV ratio, it can be concluded that digestion of unglycosylated and glycosylated CMP throughout the action of exopeptidases was similar whereas the activity of endopeptidases on GMPs was limited, probably by steric hindrance due to glycosylations. The concentration of either free amino groups or FAAs decreased from 8 to 24 h of digestion, suggesting that some BBMV enzymes are able to degrade the FAAs. It is of note that 8 or 24 h of digestion is not necessarily representative for a digestion process in vivo because the BBMV/substrate ratio has been evaluated to monitor the digestion kinetics to better understand the hydrolysis mechanism.

An interesting result was that the glycosylated forms of CMP were twice as slow and less digested than the unglycosylated ones (**Figures 1** and **2B**). Our original results showed that the glycosylation core as well as the number of the attached

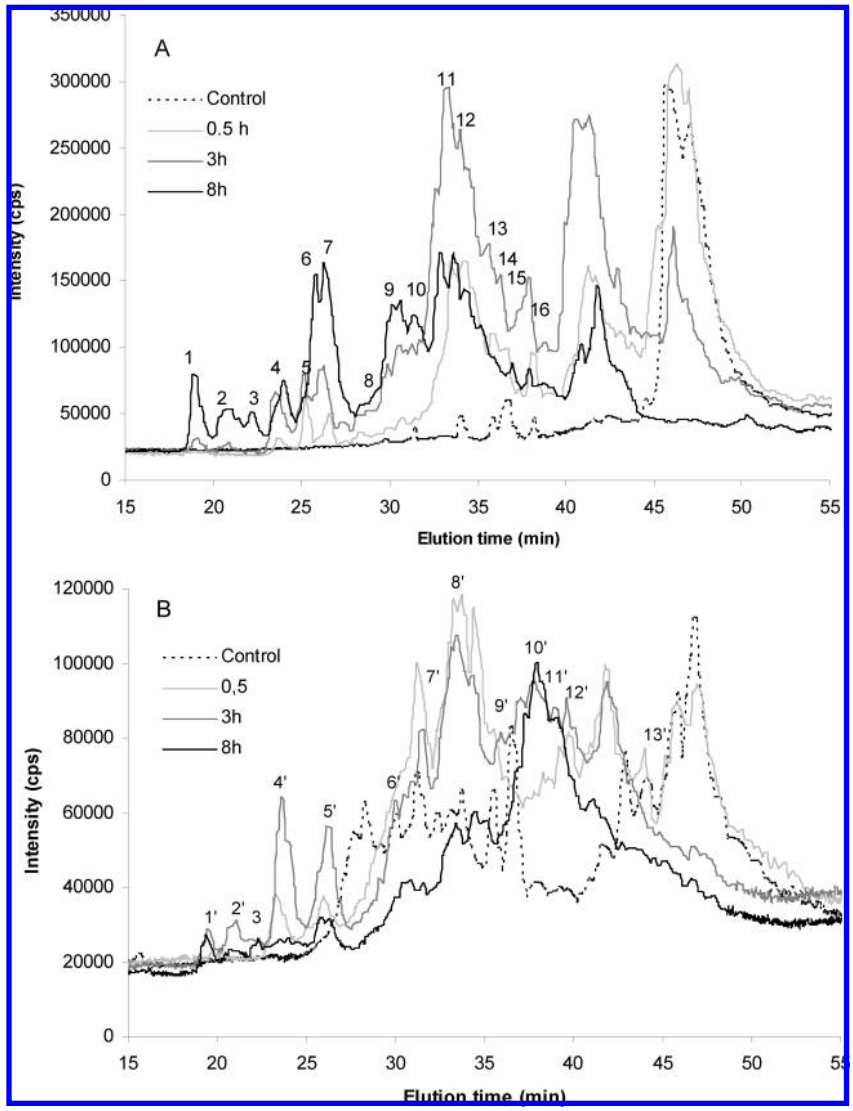

Figure 4. TIC profiles from CMP0 (**A**) and GMPs (**B**) digests obtained by nano-RP-HPLC-ESI-MS/MS after 0.5, 3, and 8 h of digestion by BBMVs and used to identify peptides (see **Tables 2** and **3**).

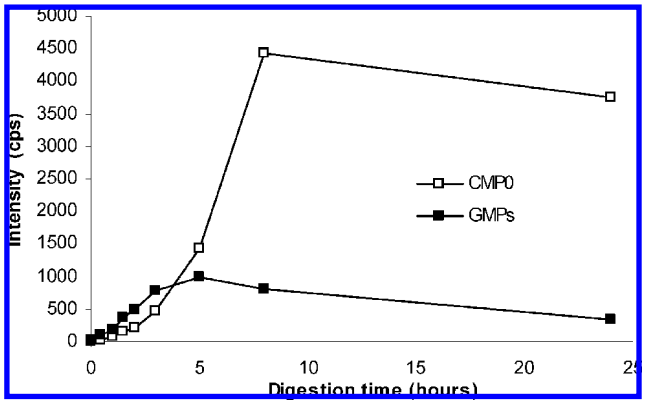

Figure 5. Intensity of the peptide κ-CN (115−121) in the hydrolysate from CMP0 (□) and total GMPs (■) throughout the digestion.

oligosaccharide modified the digestion kinetic, the most heavily glycosylated forms being the slowest digested (**Figure 3**). This suggested that BBMV enzymes were unable to hydrolyze the oligosaccharide chain of CMP sequence. Among the digestive enzymes identified from human intestinal BBM (*31*) or the mouse jejunal BBMV (*32, 33*), sucrase-isomaltase, lactase/phlorhizin hydrolase, or its precursors lactase-glycosylceramidase and trehalase are able to hydrolyze glucosidic linkages of some oligosaccharides produced from polysaccharides such as starch and glucogen. Henry-Vitrac et al. (*30*) have shown that these intestinal enzymes deglycosylated the *trans*-piceid (3-β glucoside of *trans*-resveratrol) in *trans*-resveratrol, a phenolic compound found in wine and reported to have a potential cancer chemopreventive activity (*34*). We hypothesize that for CMP, *O*-glycosylations with neuraminic acid at the end position that are linked to the sequence cannot be released because BBMs do not possess enzymes such as neuraminidase able to release them. Other posttranslational modifications such as phosphorylations also present on GMPs were rapidly released through the action of alkaline phosphatase (**Figure 3**). Similar digestion of phosphorylated casein has been previously shown (*35*); thus, phosphorylations did not alter CMP digestion as glycosylations did.

Fewer peptides were identified from GMPs digestion than from CMP0 (**Tables 2** and **3**). Moreover, in contrast to CMP0, some peptides obtained from GMPs were never digested, certainly because some peptide bonds were inaccessible to any peptidases due to the presence of attached oligosaccharides. These results take into account the peptides identified, that is, all those that were over the detection limit of the experimental setup used. Maybe others, especially some glycosylated peptides present in very small amounts, were not identified. Indeed, an

Table 2. Peptides Identified Throughout the Digestion of CMP0[a]

		sequence assignment of the peptide					sequence assignment of the peptide		
peak no.	observed molecular mass (Da)	0.5 h	3 h	8 h	peak no.	observed molecular mass (Da)	0.5 h	3 h	8 h
16	2311.13		**148**−169		11	3811.84	106−**141**	106−**141**	106−**141**
16	2196.11	149−169	149−169		9	2678.40		108−**132**	108−**132**
15	2012.04	151−169	151−169		9	2565.29			109−**132**
14	1882.98		152−169		9	2478.28		109−131	109−131
14	2141.03	**148**−167	**148**−167		9	2591.35		108−131	108−131
14	2026.00	149−167	149−167		8	2094.12		109−127	109−127
13	1783.92		152−168		6	2007.10		109−126	109−126
9	1541.79			155−169	9	2409.28		106−**127**	106−**127**
9	1371.70	155−167			10	2322.27	106−126		
14	1284.65		156−167		10	2466.32		106−128	106−128
12	1712.87	152−167	152−167		9	2264.21		108−128	108−128
13	1841.92		151−167		9	2151.14		109−128	109−128
13	1752.83	**148**−163	**148**−163	**148**−163	8	2138.39	106−**124**		106−**124**
6	983.49		155−163		6	1936.05	108−**124**	108−**124**	108−**124**
13	896.44		156−163		5	1822.96		109−**124**	
5	787.36		**148**−154		1	1607.86		108−**121**	108−**121**
8	658.32	**148**−153	**148**−153		1	1393.74			109−120
8	787.35		147−153		1	1494.81		109−**121**	109−**121**
2	669.33		156−161		2	1044.51		113−**121**	113−**121**
3	756.37	155−161	155−161	155−161	8	1372.68			113−**124**
8	998.48		153−161		9	1130.58		**115**−**124**	**115**−**124**
10	1324.68		152−163		2	802.42			**115**−**121**
9	1097.56	152−161	152−161		14	2788.33		**115**−**141**	**115**−**141**
10	2591.35		151−161		13	1458.75			**115**−128
11	1525.72		**148**−161		12	1401.72			**115**−127
16	1453.73		151−163		8	544.29			120−**124**
6	1018.50		160−169		8	657.36			119−**124**
6	904.49	161−169	161−169	161−169	17	1889.91		**128**−146	
15	704.38	163−169	163−169		8	1560.75		124−139	
17	4525.29	106−**148**	106−**148**		6	1675.78			**125**−**141**
17	4410.20		106−**147**		7	1459.70		**125**−139	**125**−139
17	4323.13		108−**148**		1	1089.50	**128**−138	**128**−138	**128**−138
17	4281.15	106−146	106−146		1	1018.55			**128**−137
17	4079.09		108−146	108−146	4	1188.54	**128**−139	**128**−139	**128**−139
12	3579.81		106−139	106−139	4	1131.55	129−139	129−136	129−139
11	3448.78			107−139	5	616.29			134−139
11	3264.65		109−139	109−139	8	1305.62		**133**−145	**133**−145
10	2880.49	106−**132**			8	1204.59			**133**−144
10	3593.80		108−**141**		4	832.38		134−**141**	
10	3480.73		109−**141**	109−**141**	9	818.43		139−146	
10	2793.46		106−131	106−131	9	889.48		138−146	
10	3506.78		108−140		17	1662.79			133−148
11	3377.71	108−139	108−139		16	1418.71		133−146	133−146
5	1835.02			108−123	16	1034.47			133−142
11	3708.84	106−140	106−140		17	1911.88			128−142

[a] The peak numbers correspond to those indicated in the TIC profiles in **Figure 5A**. The peptides generated through the supposed action of endopeptidases are pointed out with N- and C-terminal amino acid residues in bold number.

important technical limit must be commented with respect to identification by MS of the peptides obtained from glycoprotein hydrolysis. Knowing that five oligosaccharides were reported for GMP (*26*) and that the number of glycosylation sites is between 0 and 7 (*1, 2*), many different glycoforms were potentially present in our GMPs fraction. If each of these forms were hydrolyzed 10 times by the BBMV enzymes, hydrolysis of GMPs can generate many different molecular mass species. Adding the 1+ to 3+ multiple charged ions produced by ESI, the molecular mass species will lead to more than 1000 different *m*/*z* ions, therefore dividing these species concentrations. Thus, this experimental setup may not lead to the production of these ions with a high enough intensity for them to be detected.

To express their biological activities in vivo, peptides derived from CMP need to be stable within the intestine. The identification of the κ-CN (115−121) peptide in hydrolysate obtained from both CMP0 and GMPs digested for 24 h suggests that this peptide was resistant to the proteolytic action of BBMV enzymes. However, this peptide might be produced in small quantities because it was only detected by the highly sensitive technique ESI/MS. This peptide and the κ-CN (155−161), which was also identified until 8 h of digestion in both hydrolysates, DKTEIPT and SPPEINT, respectively, are two seven amino acid sequences that contain three homologue residues. The remarkable stability of these peptides in the intestinal environment suggests that they might preferentially be active in vivo in the intestinal tract as compared to the fast hydrolyzed and/or absorbed peptides.

CMP and peptides derived, as all food substances, might modulate intestinal functions such as the paracellular absorption of bioactive peptides, transepithelial transport, and BBM enzyme activities (*36*). A better knowledge of the luminal digestion of proteins and peptides is essential to identify in vivo the production of bioactive peptides, the stability of the peptides in the lumen, and the way they interact with the intestinal tract. In this view, our study on the intestinal digestion of CMP allows us to better understand its mechanisms of action and thus to gain control over its own bioactivity and the one of the peptides derived from it. Using in vitro simulated gastrointestinal digestion, Luz Sanz et al. (*37*) have shown that the products digestion yield of glycated β-lactoglobulin was lower than that observed for the unglycated protein. Although β-lactoglobulin was *N*-glycosylated while CMP was *O*-glycosylated, it can be assumed that all along the gastrointestinal tract, glycoproteins

Table 3. Peptides Identified Throughout the Digestion of GMPs[a]

peak no.	observed molecular mass (Da)	sequence assignment of the peptide 0.5 h	3 h	8 h
12′	2311.10		**148**−169	
12′	2196.10	149−169	149−169	
9′	2025.99	149−167		
10′	1882.98	152−169		
7′	2489.09		155−169[E]	155−169[E]
8′	1541.77	155−169	155−169	
6′	1371.68	155−167		
5′	983.47	155−163		
3′	756.36	155−161	155−161	155−161
11′	2012.01	156−168[C/D]	156−168[C/D]	
5′	1018.52	160−169		
5′	904.47	161−169	161−169	
4′	803.42	162−169	162−169	
12′	704.37	163−169		
13′	2780.20			129−146[E]
4′	1131.55		129−139	
4′	1188.51	**128**−139	**128**−139	
1′	1089.44		**128**−138	
5′	1823.18			109−**124**
5′	1936.01			108−**124**
2′	802.39		**115**−**121**	**115**−**121**
2′	1044.50			113−**121**
1′	1494.76		109−**121**	109−**121**
2′	1607.85			108−**121**

[a] The peak numbers correspond to those indicated in the TIC profiles in **Figure 5B**. When the peptide was glycosylated, the nature of the attached carbohydrate chain was mentioned using letter as reported in **Table 1**. The peptides generated through the supposed action of endopeptidases are pointed out with N- and C-terminal amino acids residues in bold number.

are less digested than their unglycosylated homologue protein. As a consequence, glycosylation could represent a protective mechanism for food-derived bioactive peptides such as CMP.

ABBREVIATIONS USED

BBM, brush border membrane; BBMV, brush border membrane vesicles; CMP, caseinomacropeptide; ESI, electrospray ionization; FAAs, free amino acids; GMP, glycomacropeptide; κ-CN, κ-casein; MS, mass spectrometry; TIC, total ion current; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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