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Sequential release of milk protein–derived bioactive peptides in the jejunum in healthy humans[1–4]

Rachel Boutrou, Claire Gaudichon, Didier Dupont, Julien Jardin, Gheorghe Airinei, Agnès Marsset-Baglieri, Robert Benamouzig, Daniel Tomé, and Joëlle Leonil

ABSTRACT

Background: The digestive hydrolysis of dietary proteins leads to the release of peptides in the intestinal tract, where they may exert a variety of functions, but their characterization and quantification are difficult.

Objectives: We aimed to characterize and determine kinetics of the formation of peptides present in the jejunum of humans who ingested casein or whey proteins by using mass spectrometry and to look for and quantify bioactive peptides.

Design: Subjects were equipped with a double-lumen nasogastric tube that migrated to the proximal jejunum. A sample collection was performed for 6 h after the ingestion of 30 g ^{15}N-labeled casein (n = 7) or whey proteins (WPs; n = 6). Nitrogen flow rates were measured, and peptides were identified by using mass spectrometry.

Results: After casein ingestion, medium-size peptides (750–1050 kDa) were released during 6 h, whereas larger peptides (1050–1800 kDa) were released from WPs in the first 3 h. A total of 356 and 146 peptides were detected and sequenced in the jejunum after casein and WP ingestion, respectively. β-casein was the most important precursor of peptides, including bioactive peptides with various activities. The amounts of β-casomorphins (β-casein 57–, 58–, 59–, and 60–66) and β-casein 108–113 released on the postprandial window were sufficient to elicit the biological action of these peptides (ie, opioid and antihypertensive, respectively).

Conclusions: Clear evidence is shown of the presence of bioactive peptides in the jejunum of healthy humans who ingested casein. Our findings raise the question about the physiologic conditions under which these peptides can express their bioactivity in humans. This trial was registered at clinicaltrials.gov as NCT00862329. *Am J Clin Nutr* 2013;97:1314–23.

INTRODUCTION

Protein digestion is a complex process that involves dynamic movements and exchanges of proteins, peptides, amino acids, urea, and ammonia between the gut lumen and systemic pools. After their ingestion, dietary proteins are mixed with almost an equal quantity of endogenous protein voided into the digestive tract (1). This protein material is hydrolyzed by luminal gastric and pancreatic enzymes and epithelial brush border membrane peptidases into a complex mixture of oligopeptides, dipeptides, tripeptides, and free amino acids that transit and are progressively absorbed along the small intestine. Different studies have suggested that bioactive peptides released from dietary protein during digestion could exert metabolic and physiologic actions by acting on specific targets at the digestive level or after absorption (2–5), and some studies have particularly identified potential bioactive peptides in the sequence of milk proteins (6).

In vitro studies have been performed to determine peptides released throughout simulated gastrointestinal digestion. However, peptides produced by in vitro digestion can differ from those generated in vivo because the environmental conditions of in vivo digestion can hardly be reproduced such as the enzyme:substrate ratio, reaction time, and pH, which all control digestion variables and change through in vivo digestion (7). In addition, in vivo digestion can be considered dynamic phenomena that result from a continuous delivery of gastric effluents in the small intestine, with the intestinal absorption of some nutrients in the upper part of the tract while others continue to the distal part. To understand the metabolic and physiologic consequences of the interaction between dietary proteins and the gastrointestinal tract, the effective in vivo release of peptides has to be established. Only scarce in vivo studies have been undertaken to identify peptides released by digestion in animals (8, 9), human (10, 11), and both (12, 13), despite promoting outcomes of this topic.

Accordingly, this study aimed to characterize digestion products and, particularly, peptides released in the jejunum after ingestion of

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either milk casein or whey proteins (WPs)[5] in healthy human subjects. We used a gastrointestinal tube technique to collect effluents as well as mass spectrometry (MS) to extensively identify and quantify milk protein–derived peptides.

SUBJECTS AND METHODS

Production of milk proteins

Milk proteins were intrinsically labeled with ^{15}N, purified, and prepared as previously described (14). Briefly the milk was defatted and microfiltered on a 1.4-μm membrane. The permeate was concentrated and microfiltered on a 0.1-μm membrane, which allowed the separation of micellar caseins in the retentate and WPs in the permeate; the latter was concentrated further by ultrafiltration on a 5-kDa membrane. Each fraction was lyophilized and analyzed for microbiological quality.

Participants and experimental design

All participants were certified as being in good health after a thorough examination performed by the medical staff of the Human Nutrition Research Centre of Avicenne Hospital and routine biochemical tests. Eligibility criteria were as follows: negative serology for HIV and hepatitis B and C; the absence of any pathology; the absence of an allergy to dairy proteins; pregnancy or absence of contraception for women; BMI (in kg/m^2) <30; and age 18–40 y. The purpose and potential risks of the study as well as constraints because of the obligatory dietary standardization period were fully explained to subjects. Written informed consent was obtained from all participants, and the protocol was approved by the Institutional Review Board of Saint-Germain-en-Laye Hospital. This trial was registered at clinicaltrials.gov as NCT00862329 (SURPROL).

The study was single-blinded trial and was conducted by using a 2-arm parallel design. Twenty-six subjects were initially recruited from March 2008 to June 2010. The recruitment was stopped when 8 exploitable kinetics were obtained in each group. Regarding previous experiments on digestive kinetics (15–17), this size sample was considered to be sufficient to discriminate between groups. Because of various difficulties with the tolerance of the nasojejunal tube (painful, vomiting, and nonmigration through the pylorus), the study was finally performed in 16 subjects [10 men and 6 women; BMI (mean ± SD): 28.3 ± 1.8; age: 30 ± 6 y] (**Table 1**). Subjects were alternately allocated to either a casein or whey group to avoid any random effect as a result of the season (such as the usual diet of the subject). However, during the last phase of the study, sex, age, and BMI were used as criteria to homogenize groups.

Standardization diet and experimental meal

The diet of subjects was standardized during 9 d to achieve a protein intake of 1.4 g · kg^{-1} · d^{-1}, which corresponded to the average protein intake of the French population; *see* Table S1 under "Supplemental data" in the online issue for an example of the dietary notebook. The daily protein intake of subjects included a habituation to their protein supplement, which comprised 30 g casein or WPs (Ingredia), 27.5 g maltodextrine (Roquette), and 2.5 g orange flavor. At the beginning of the standardization, each subject was given 7 shakers that contained the supplement powder for self-administration after dissolution in 500 mL H_2O. The experimental meal was the same as the daily supplement, but proteins were intrinsically labeled with ^{15}N. Subjects were not aware of the nature of the protein they received.

TABLE 1
Characteristics of subjects

	Casein (n = 8)	Whey protein (n = 8)
Sex (no. M, no. F)	5, 3	5, 3
Age (y)	32 ± 6[1]	28 ± 7
Weight (kg)	85 ± 8	86 ± 7
BMI (kg/m^2)	28.5 ± 2.1	28.1 ± 1.3

[1] Mean ± SD (all such values).

Jejunal-sample collection and preparation

On the eighth day, subjects came to the Human Nutrition Research Centre of Avicenne Hospital to achieve a digestive exploration. Subjects were equipped with a double-lumen nasogastric tube that migrated to the proximal jejunum, as previously described (17). The location of the sampling site was controlled by using radiography. On the ninth day, after subjects fasted overnight, a solution of polyethylene glycol 4000, which was used as a nonabsorbable marker, was infused through the intestinal tube.

A basal collection of jejunal effluents was performed during 0.5 h, after which subjects were asked to ingest their test meal as previously described. Sample collection was performed for 6 h after the ingestion of the test meal. Jejunal effluents were continuously collected on ice and pooled every 30 min. The effluents were freeze-dried until analysis. The concentration of polyethylene glycol 4000 in digesta samples was determined by using a turbidimetric method (18) to determine the liquid flow rate. The total nitrogen in the digesta was determined by using an elemental nitrogen analyzer (Euro Elemental Analyzer 3000; EuroVector) with atropina as a standard. The ^{15}N:^{14}N isotope ratio was determined by using isotope-ratio MS (Optima; Fisons Instruments). The atom percent excess (APE) of samples were calculated by subtracting the baseline value from the atom percentage determined at each time point. The nitrogen flow rate was calculated as follows:

$$\text{Total } N\,(\text{mmol}) = \text{flow}\,(\text{mL}) \times N\,(\%) \times \text{DM}\,(\%) \div 14 \quad (1)$$

where flow is the liquid flow rate, N is the age 18–40 y nitrogen in the sample expressed as percentage, DM is the dry matter, and 14 is the nitrogen molar mass. The dietary nitrogen flow rate was calculated as follows:

$$\text{Dietary } N\,(\text{mmol}) = \text{Total } N\,(\text{mmol}) \times \text{APE sample} \div \text{APE meal} \quad (2)$$

where APE sample and APE meal are the ^{15}N enrichments in the digesta and meal, respectively. For additional protein and peptide analysis, 0.1 g freeze-dried samples were solubilized at 0.1 g/mL in 50 mmol tris-HCl buffer/L (pH 8.0) and 8 mol urea/L, which were hardly stirred and incubated at 40°C for

[5] Abbreviations used: ACE, angiotensin-converting enzyme; APE, atom percent excess; BSA, bovine serum albumin; IC_{50}, half maximal inhibitory concentration; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TFA, trifluoroacetic acid; WP, whey protein; XIC, extracted ion chromatogram.

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1 h to dissolve the freeze-dried digest. The samples were centrifuged for 10 min at 10,000 × *g*. A total of 300 μL supernatant fluid was diluted in 900 μL 50 mmol Tris-HCl buffer/L (pH 8.0) to a 25-g/L final concentration and filtered through a 0.45-μm filter (Minisart). Samples were frozen at −20°C until analysis.

Identification of peptides by using tandem mass spectrometry

All mass spectra were performed by using a hybrid quadrupole time-of-flight mass spectrometer (QStar XL; MDS Sciex). The instrument was calibrated with a multipoint calibration by using fragment ions that resulted from the collision-induced decomposition of a peptide from β-casein (193–209) (NeoMPS SA). After a 1:200 dilution in 0.1% trifluoroacetic acid (TFA) (Pierce, Touzart et Matignon), the peptide fraction (10 μL) was trapped onto a C_{18} PepMap 100 microprecolumn cartridge (300-μm inner diameter × 5 mm; Dionex) before the separation of peptides onto a C_{18} PepMap 100 column (75-μm inner diameter × 150 mm; Dionex). The separation started with 5% solvent B for 5 min, and a linear gradient from 5% to 70% of solvent B for 65 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 liquid chromatography–grade water; and solvent B contained 95% acetonitrile, 0.08% formic acid, and 0.01% TFA in liquid chromatography–grade water. Online separated peptides were analyzed by using electrospray ionisation on a quadrupole time-of-flight mass spectrometer in positive-ion mode. An optimized voltage of 3.2 kV was applied to the nanoelectrospray ion source (Proxeon Biosystems A/S). MS and tandem mass spectrometry (MS/MS) data were acquired in continuum mode. Data-direct analysis was used to perform MS/MS analysis on 1^+- to 4^+-charged precursor ions. Precursor selection was based on ion intensity and charge state, and if the precursors had been previously selected for fragmentation, they were excluded for the rest of the analysis. Spectra were collected in the selected mass range from 400 to 1500 *m/z* for MS spectra and from 60 to 2000 *m/z* for MS/MS. The mass spectrometer was operated in data-dependent mode, which automatically switched between MS and MS/MS acquisition with Analyst QS 1.1 software (Applied Biosystems) when the intensity of the ions was >10 counts/s. To identify peptides, all data (MS and MS/MS) were submitted to MASCOT v.2.2 software (Matrix Science). The search was performed against a homemade database that deals with major milk proteins that represent a portion of the Swissprot database (http://www.uniprot.org); consequently, endogenous proteins were not identified in the current study. No specific enzyme cleavage was used, and the peptide mass tolerance was set to 0.2 Da for MS and 0.15 Da for MS/MS. For each peptide identified, a minimum MASCOT score that corresponded to $P < 0.05$ was considered as a prerequisite for peptide validation. A second consecutive run was performed from the same sample vial to perform a single MS data collection. This method ensured that enough time points would be observed for the quantification of peptides via extracted ion chromatograms (XICs).

Identification of bioactive peptides

A search for the biological activity of peptides identified was conducted through the Dziuba database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php) and by comparing sequences either reviewed or described in numerous articles (19–36).

Quantification of some bioactive peptides

Synthetic peptides were solubilized at 5 g/L in 35 mmol HEPES-tris buffer/L, which was diluted first 1:1000 with 0.1% TFA to obtain the 5-mg/L solution. An 8-point calibration was carried out with solutions of β-casein (60–66) (10–1000 pg) and β-casein (108–113) (10–5000 pg). Quantification was performed using the Analyst QS 1.1 software. Peptide quantity was measured from the XIC obtained for each peptide. *m/z* ranges of 790.4 ± 0.3 Thomson and 748.39 ± 0.3 Thomson were extracted for β-casein (57–, 58–, 59–, and 60–66) and β-casein (108–113), respectively. The peak maximum intensities were considered to construct the calibration curve. The β-casein (60–66) and β-casein (108–113) quantity (*x*) in jejunal effluent was calculated by using the equation

$$y = bx + c \quad (3)$$

where *y* is the maximal intensity measured on the XIC, *c* is the *y*-axis intercept, and *b* is the slope of the standard curve of the peptide. The equations of calibration curves were

$$y = 14.721x + 194.94 (R^2 = 0.9938) \quad (4)$$

and

$$y = 3.1187x + 5.3819 (R^2 = 0.999) \quad (5)$$

for β-casein (57–, 58–, 59–, and 60–66) and β-casein (108–113), respectively. With respect to the quantification of β-casein (108–113), the XIC of the peptide in most jejunal effluents was higher than the maximum of our calibration curve; consequently, quantifications were extrapolated from the calibration curve, which resulted in a potential underestimation of this peptide quantity.

Calculation of the frequency of the identified peptide

The frequency of a peptide represents the number of times that it was identified in samples from all subjects fed the same meal and throughout the digestion. The frequency was calculated as the ratio of the number of times the peptide was identified over the number of times the peptide would be identified if present throughout digestion (12-sample collection for casein and 9-sample collection for WPs because no peptide was identified after 5 h) and for all subjects fed the same meal ($n = 7$ for casein; $n = 6$ for WPs). Thus, a frequency of 1 meant that the peptide had been identified in all effluents whatever the digestion time and subject. Peptides with a frequency ≥0.60 were named frequent peptides.

Statistics

For nitrogen flow rates, the effect of treatment was assessed by using a mixed model with the group as a simple factor and time as a repeated factor with the MIXED procedure in SAS software

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(version 6.11: SAS Institute Inc). $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of jejunal effluents

The kinetics of dietary nitrogen flux in the jejunal effluents differed between casein and WP groups (time × group: $P < 0.0001$) (**Figure 1**). The delivery of dietary nitrogen was massive after WPs and was completed after 3 h, whereas casein-derived dietary nitrogen was progressively emptied from the stomach for 6 h postmeal. In contrast, the flux of endogenous nitrogen remained relatively stable during the kinetic (5–12 mmol N/30 min) and was not different between groups (Figure 1). After 6 h, the jejunal absorption of dietary nitrogen was 62 ± 9% for casein and 50 ± 7% for WPs and was, thus, not significantly different. The size distribution of peptides detected in jejunal effluents differed after the ingestion of casein and WPs (**Figure 2**). Peptides were classified into 4 groups according to their molecular weight as follows: 450–750, 750–1050, 1050–1800, and 1800–3000 Da. For the casein group, most of the peptides had a molecular weight that ranged from 450 to 1800 Da. The 750–1050-Da peptides (6–9 amino acid residues) were the most predominant throughout the 6-h digestion, whereas the number of 1050–1800-Da peptides started to decrease after 3 h of digestion. In the WP group, the predominant frequency of 1050–1800-Da peptides (9–15 amino acid residues) was observed throughout the digestion. Almost no peptide was detected 4.5 h after the meal in this group.

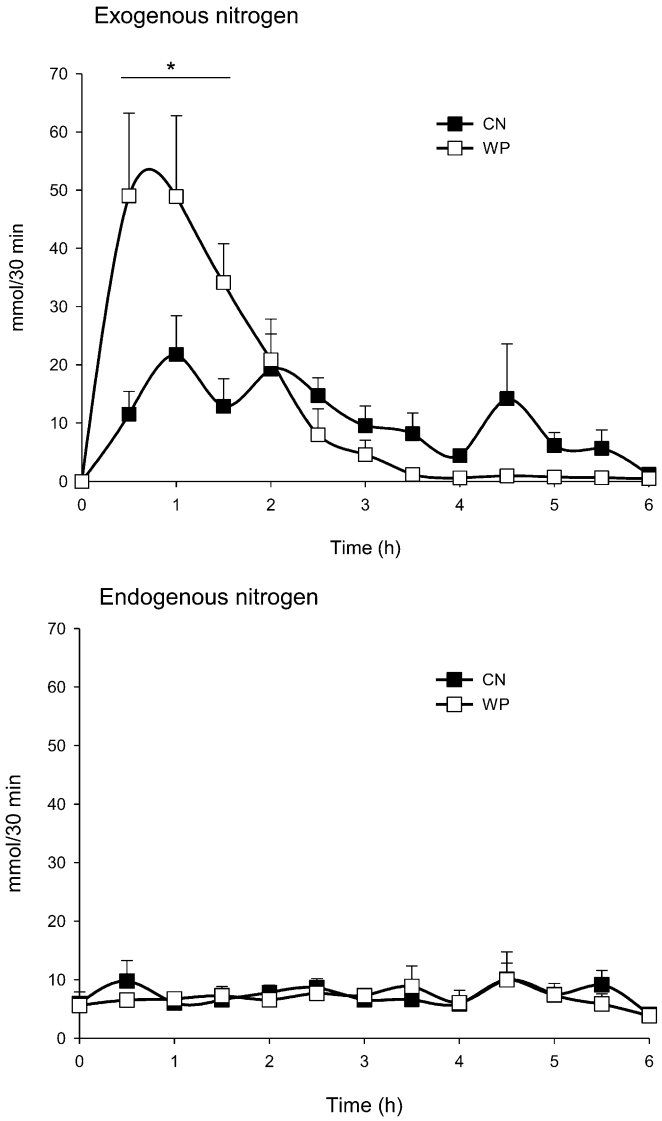

FIGURE 1. Mean (±SD) contributions of exogenous nitrogen and endogenous nitrogen to total nitrogen in the jejunum of subjects after the ingestion of CN or WP (n = 8 per group). Kinetics differed between groups (mixed-model analysis; time × group: $P < 0.0001$) *Significantly different at each time point, $P < 0.05$ (post hoc test). CN, casein; WP, whey proteins.

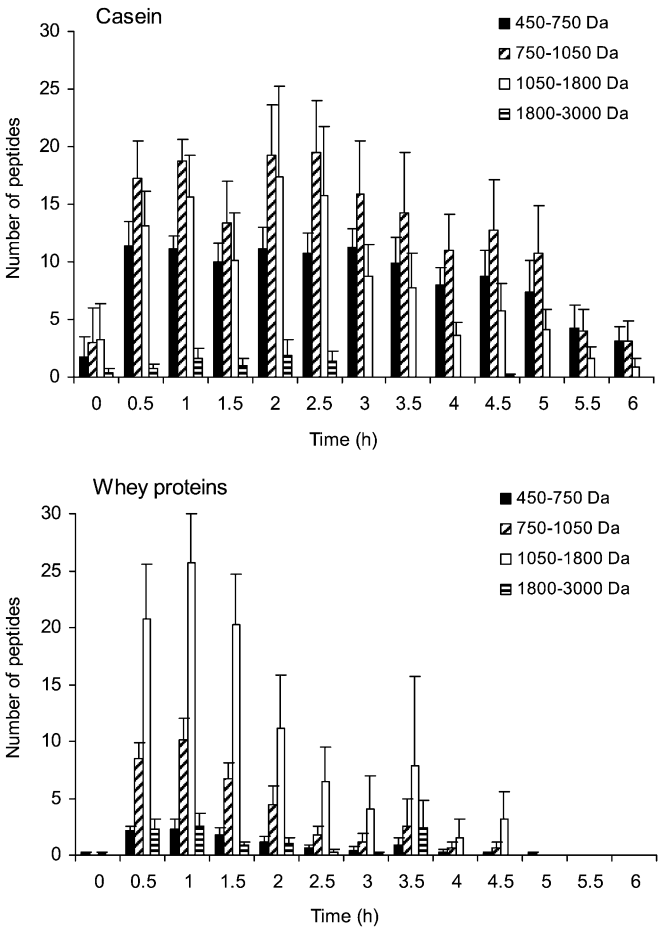

FIGURE 2. Mean (±SD) numbers of peptides in the jejunum of subjects after the ingestion of casein (n = 7) and whey proteins (n = 6). Peptides are classified according to their molecular weight.

Identification of casein-derived peptides

Within jejunal effluents collected throughout the 6-h digestion, 356 casein-derived peptides were identified including 218 peptides from β-casein (61.2%) and 85 peptides from α_{s1}-casein (24.9%) whereas the other α_{s2}- and κ-casein–derived peptides accounted for 8.2% and 6.7%, respectively. For β-casein, no peptide was shown in the 2 sequences 15–48 and 92–105, whereas other peptides were recovered in effluent samples from all subjects that included 11 β-casein–derived peptides with a frequency >0.60 (*see* Table S2 under "Supplemental data" in

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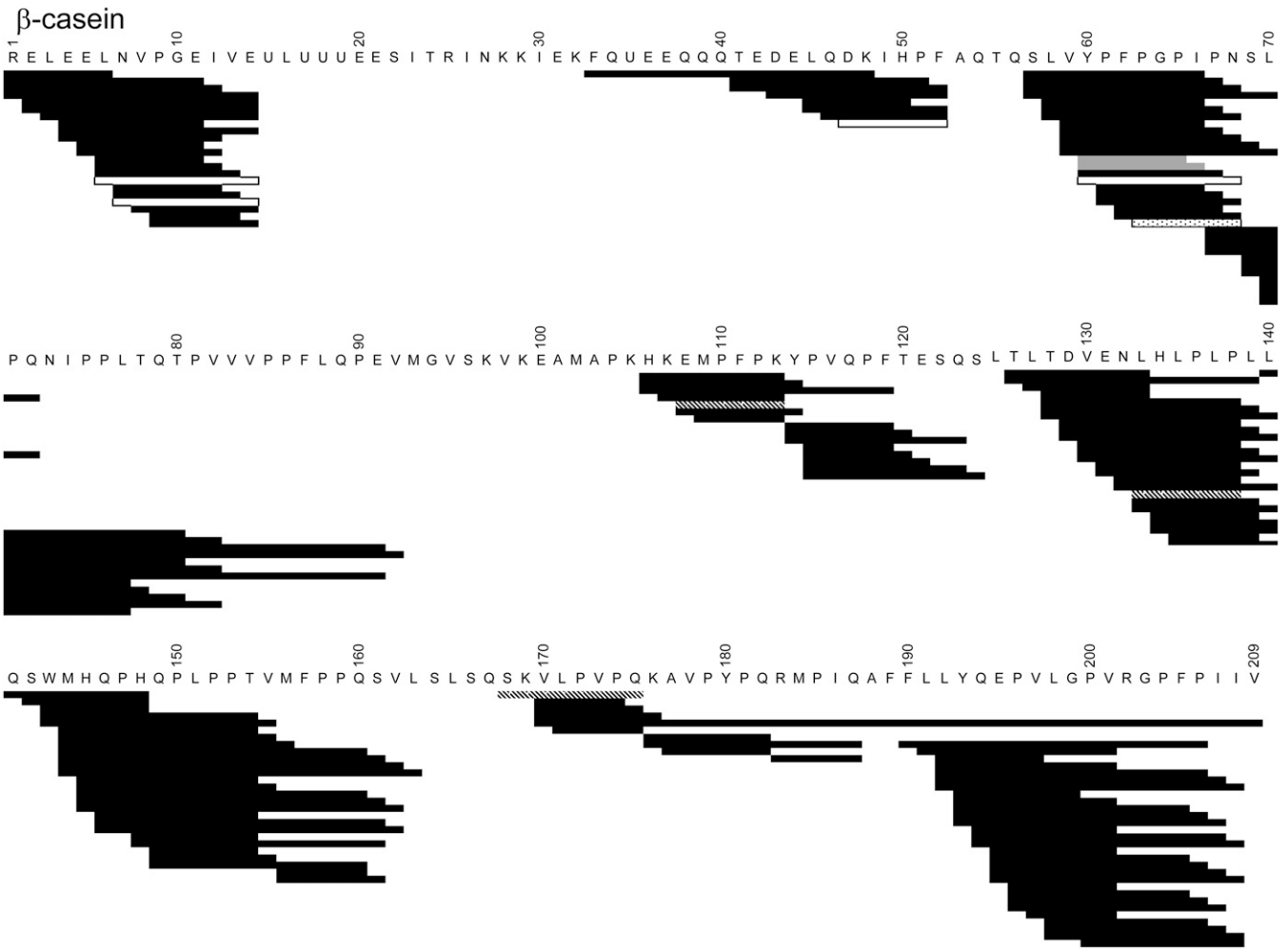

FIGURE 3. Milk peptides released from β-casein that were identified by using liquid chromatography–tandem mass spectrometry in jejunal effluents of humans fed casein (n = 7). Peptides having a biological activity are presented as angiotensin-converting enzyme inhibitory peptides (open bars), opioid peptides (gray bars), immunomodulary peptides (dotted bar), and antihypertensive peptides (left diagonally hatched bars).

the online issue) with 4 derived from the β-casein (57–68) sequence (**Figure 3**). In addition, 112 peptides were identified with a frequency <0.05 (ie, were identified in <5% of analyzed samples), in which 12 bioactive peptides were included. The most-frequent bioactive peptides were β-casomorphins β-casein (59–66), β-casein (59–68), and β-casein (60–66), the antihypertensive β-casein (108–113) peptide, and other bioactive peptide precursors including 27 and 32 precursors for the antihypertensive tripeptides IPP (sequence 74–76) and VPP (sequence 84–86) (30), respectively. The α_{s1}-casein (24–30) peptide was the only frequent α_{s1}-casein–derived peptide (frequency: 0.65), and 45% of the most frequently identified peptides originated from sequence 4–33, which counted many precursors of bioactive peptides (**Figure 4**). The following 3 bioactive peptides were identified from α_{s1}-casein: α_{s1}-casein (24–32) and α_{s1}-casein (104–109), which have an angiotensin-converting enzyme (ACE)–inhibitory activity, and mineral carrier α_{s1}-casein (106–119). Most of the 29 peptides identified from α_{s2}-casein were released from the 89–122 sequence, whereas no peptides within the 1–24 sequence were identified, and only 2 peptides originated from the C-terminal end of α_{s2}-casein. Also, few peptides were identified from the κ-casein, with the most frequent peptides identified from the 52–59 sequence. No peptides were identified from the N-terminal end of κ-casein, from the 60–105 sequence, and the C-terminal end. Two peptides with an antithrombotic activity were shown within the 106–116 sequence.

Identification of WP-derived peptides

Within the jejunal effluents collected from all subjects throughout the 6-h digestion, 146 peptides originated from WPs, including 105 peptides from β-lactoglobulin (72%), whereas percentages of peptides identified from α-lactalbumin, bovine serum albumin (BSA), and lactoferrin represented 15.1%, 11.0%, and 1.4%, respectively. The 105 peptides identified from β-lactoglobulin mainly originated from 40–58 and 122–137 sequences, which seemed to be sequentially hydrolyzed through the action of peptidases (**Figure 5**). In contrast, few peptides were shown in sequences 1–41, 60–82, and 100–121 of β-lactoglobulin. For α-lactalbumin, 80% of the 85 identified peptides originated from the 80–90 sequence, and no peptides from the 2 terminal ends were identified (ie, from 1–17 and 103–123 sequences). For BSA, 16 peptides were identified. For lactoferrin only, the following 2 peptides were identified: 139–145 and 607–611

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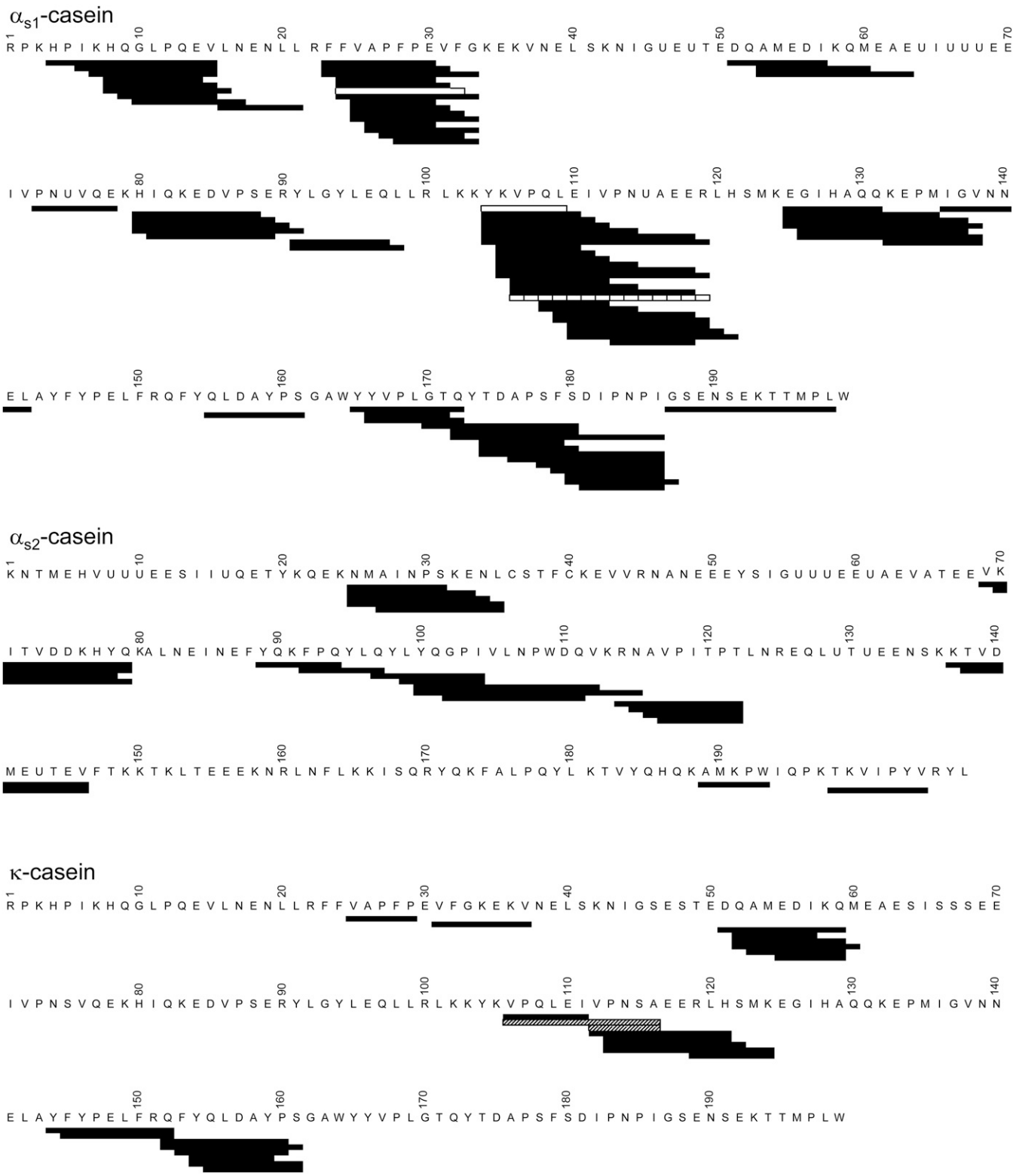

FIGURE 4. Milk peptides released from α_{s1}-, α_{s2}-, and κ-casein that were identified by using liquid chromatography–tandem mass spectrometry in jejunal effluents of humans fed casein (n = 7). Peptides having a biological activity are presented as angiotensin-converting enzyme–inhibitory peptides (open bars; α_{s1}-casein), mineral carrier (vertically hatched bar; α_{s1}-casein), and antithrombotic peptides (right diagonally hatched bar; κ-casein).

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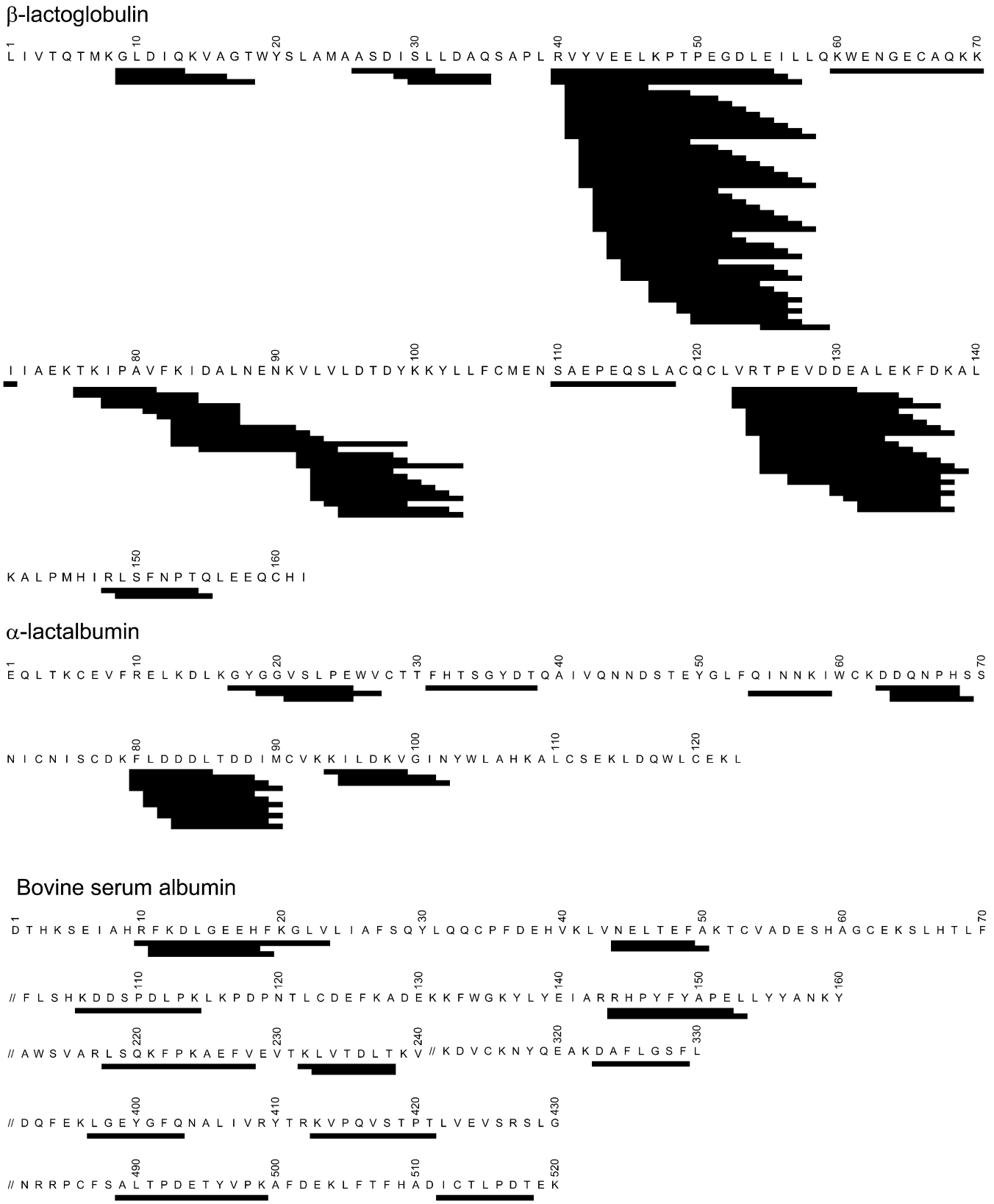

FIGURE 5. Milk peptides released from β-lactoglobulin, α-lactalbumin, and bovine serum albumin that were identified by using liquid chromatography–tandem mass spectrometry in jejunal effluents of humans fed whey proteins (n = 6).

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sequences. None of the peptides identified from WPs were bioactive peptides from data reported in the literature (19, 32, 34). However, peptides identified included 35 precursors of bioactive peptides from β-lactoglobulin and one α-lactalbumin–derived (18–20) peptide immunomodulator precursor.

There are some protein sequences for which no peptides were identified by using our experimental conditions. One possibility is that these sequences have been degraded in <5 amino acid residue peptides that were not detectable. Another possibility is that these peptides were not identified because of their large size.

Quantification of peptides β-casein (57–, 58–, 59–, and 60–66) and β-casein (108–113)

All β-casein (57–, 58–, 59–, and 60–66) and β-casein (108–113) bioactive peptides were present in the jejunal effluent throughout the 6-h digestion (**Figure 6**). Their amounts tended to be higher during the first 2 h and decreased thereafter. The quantity of the β-casein (108–113) peptide was ~20-fold higher than that of the β-casein (60–66) peptide. The cumulated quantity of the 3 precursors of the β-casein (60–66) peptide and β-casein (60–66) peptide was calculated. The quantity was the highest at 0.5 h at 3.60 ± 0.35 mg (Figure 6). There was reduction in SD by cumulated peptides, especially in the first 2 h of digestion, which suggests that cumulative analyses may be a way to solve difficulties created by the heterogeneity of the digestive process.

After 2 h of digestion, the total quantity of β-casomorphin-7 measured in jejunal effluents was 4 mg. According to the molecular weight of the peptide (789.4 Da), it was estimated that >5 μmol were available in a mean volume of jejunal effluent of 304 ± 12.6 mL (ie, a 17-μmol/L concentration). Similarly, a 40-mg mean quantity of β-casein (108–113) peptide was measured in jejunal effluent as early as at 30 min of digestion. According to the mean volume of jejunal effluent that was 59.7 ± 33.2 mL, it was estimated that 670 μg/mL (equivalent to 0.90 mmol/L) were available for in vivo activity, which was probably an underestimated quantity.

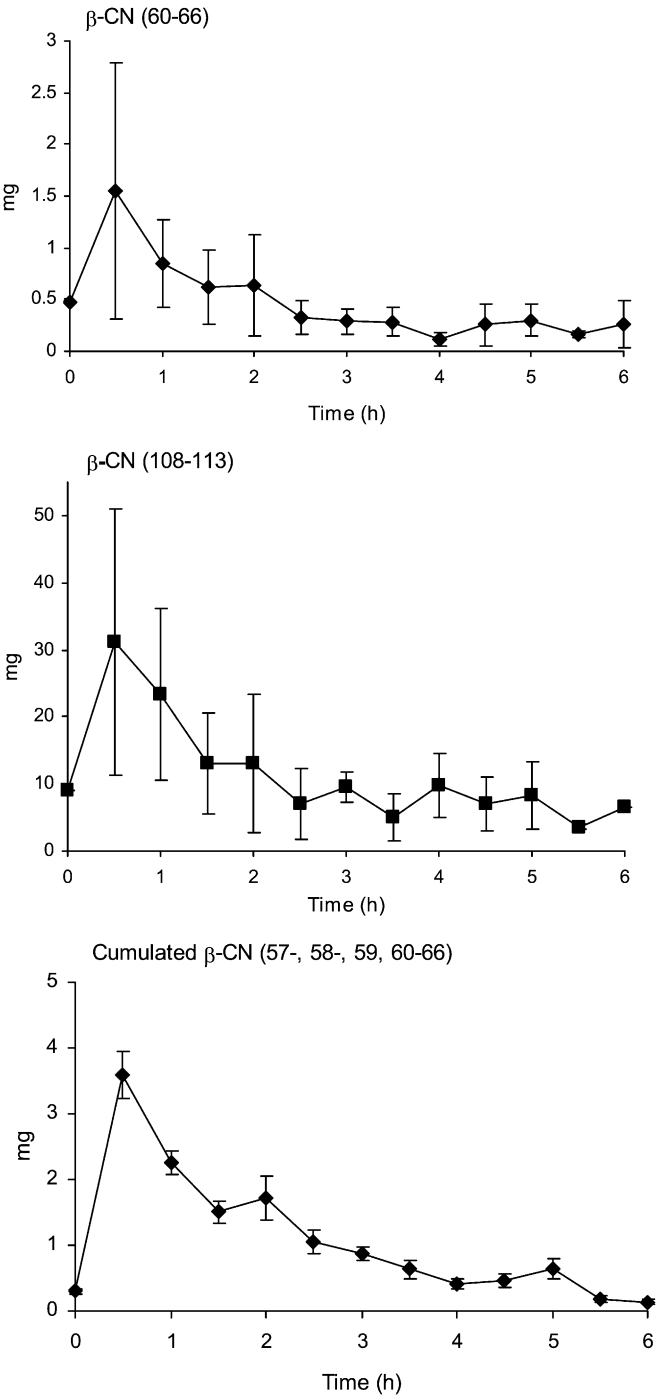

FIGURE 6. Quantification (means ± SDs) of bioactive peptides β-CN (60–66) and (108–113) and the cumulated quantity of β-CN (57–, 58–, 59–, and 60–66) as measured in jejunal effluents of humans fed casein (n = 7). CN, casein.

DISCUSSION

This study aimed to characterize kinetics of the hydrolysis of milk casein and WPs and identify peptides derived from these proteins in the jejunal effluent in healthy human subjects. The results showed that caseins released a wide range of medium-sized peptides throughout the 6 h of digestion, whereas less peptides but of larger size were released from WPs. Our work identified several peptides that have biological activities and also showed that β-casein (60–66) and (108–113) peptides, which have opioid and antihypertensive properties, respectively, were released in amounts that were compatible with a biological action.

Jejunal kinetics of dietary nitrogen are consistent with the current results on digestion rates of WPs and casein (14, 17, 37). WPs were entirely emptied from the stomach after 3 h, whereas caseins were slowly delivered all along the postprandial phase. However, differences in the gastric emptying rates did not influence the secretion of the endogenous protein that remained stable for 6 h and did not increase after the meal. The influence of dietary proteins on pancreatic secretions has been debated, and their stimulatory effect remains controversial regarding both quantitative (38, 39) and qualitative aspects (16, 40), and a mild stimulation after casein ingestion could not be excluded (15).

The recovery of dietary nitrogen indicated that 50% and 60% of ingested nitrogen had already been absorbed after 6 h at the jejunal level in WP and casein groups, respectively. Thus, the remaining nitrogen compounds were representative of the less-digestible fraction. An analysis of the degree of hydrolysis of these compounds showed that, although there was a limited

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gastric hydrolysis because of their rapid arrival in the jejunal lumen, WPs were further hydrolyzed by pancreatic and intestinal proteases, as shown by the presence of ~40 peptides 1 h after the meal. The 9–15 amino acid residue peptides were the most frequent throughout WP digestion. In contrast, the predominance of 6–9 amino acid residue peptides from casein suggested that peptides released from casein are more susceptible to hydrolysis and subsequent absorption than those from WPs. Actually, the more advanced the casein digestion was, the smaller the peptides were. The peptide-length distribution was similar to that obtained in the duodenum after ingestion of milk or yogurt in humans (10) and beef meat and trout flesh in pigs (8). Also, large peptides were sequentially sliced both to N- and C-terminal ends probably because of the action of carboxypeptidases and aminopeptidases The absence of the later in pancreatic juice (41, 42) has led to a consideration of the action of the 6 putative aminopeptidases of the brush border membrane of epithelial cells (43). These aminopeptidases are located at the surface of the epithelial cells and can be present in gastrointestinal juice that arise from sloughed-off mucosal cells (43).

Peptides present in the jejunum throughout digestion were analyzed in terms of origin and sequence. The large number of peptides identified from β- and α_{s1}-casein covered almost the whole sequence of both proteins. Few peptides from minor proteins such as κ-casein, lactoferrin, and BSA have been identified. BSA and lactoferrin are highly structured proteins that have a molecular weight 5–6 times that of other milk proteins, which may explain the few peptides identified from both these proteins. Indeed, lactoferrin was shown to be highly resistant to gastric digestion in vitro (44) and in adults (45). In addition, scarce peptides have been identified from sequences β-lactoglobulin (60–75) and β- lactoglobulin (103–123) that are protected from digestion by virtue of 4 cysteine residues located at the 66, 106, 119, and 160 amino acid residues that help to maintain the tertiary structure by forming disulfure bonds. We noticed that most of the peptides identified (eg, 81% for β-casein peptides) contained $\geq$2 proline residues, and the largest peptide identified counted 7 proline residues in sequence on 26 amino acid residues. These results were in agreement with the generally reported resistance of proline-containing peptides to gastric and pancreatic digestive enzymes (28, 46, 47) and epithelial proteases (8). In more detail, the 3 bonds Pro-Gly (63–64), Gly-Pro (64–65), and Pro-Pro (75–76; 85–86) were present in most of the peptides identified throughout digestion. Our results evidenced the predominant presence of proline-rich peptides and glycine-rich peptides in the jejunum, which questioned their role in the lumen. Recently, glycine-rich peptides with an antimicrobial activity have been reported (48).

Peptides we identified exhibited a wide range of biological functions, including antihypertensive (such as an ACE inhibitor), antithrombotic, immunomodulatory, opioid agonist and antagonist, and antimicrobial functions. As regards important activities, β-casomorphins are a family of opioid peptides derived from the (60–70) of bovine β-casein. In jejunal effluents, we identified 15 opioid sequences that overlaid the common structural motif for region 60–65 (YPFPGP). These peptides included the well-known β-casomorphin-7 (60–66) as well as large peptides of 16 amino acid residues such as β-casein (57–72). Previous studies have shown that β-casomorphin-4 (60–63), β-casomorphin-5 (60–64), and β-casomorphin-7 are released in the jejunum of minipigs fed casein (9), and considerable amounts of β-casomorphin-7, but no β-casomorphin-5, and only small amounts of β-casomorphin-4 or -6 immunoreactive materials were shown in intestinal effluents in humans after the ingestion of milk (49). We identified other numerous precursors of bioactive peptides, particularly the antihypertensive tripeptides IPP and VPP that cannot be detected under the conditions used in MS. However, 59 precursors of both tripeptides have been identified from β-casein.

The frequent identification of β-casomorphin-7 showed that it was continuously released within the lumen in humans fed casein. The 17-μmol/L amount estimated after 2 h of digestion was compatible with an in vivo opioid agonist activity because the half maximal inhibitory concentration (IC_{50}) of β-casomorphin 7 is 3–100 μmol/L for this activity (50).

As concerns the antihypertensive activity, the β-casein (108–113) is an ACE inhibitor that has an IC_{50} value of 423 μg/mL (31). The 670-μg/mL concentration estimated after 30 min of digestion was 1.5 higher than the IC_{50}. The bioavailability of ACE-inhibitor peptides also depends on their ability to be absorbed to reach nonluminal targets. It is known that small peptides such as dipeptides and tripeptides are transported by a specific transporter as shown for VPP and IPP (51, 52) whereas oligopeptides can be transported by transcytosis (52, 53) and paracellular routes (52). Whatever transport mechanism, the intestinal absorption is only 1–0.1%, which reduces the peptide bioavailability.

In conclusion, the study was conducted in a limited sample size and in subjects with an invasive method that did not allow large screening in the population. However, the profiles of hydrolysis kinetics were very repeatable in subjects and were characteristic of the protein source. The results prove the in vivo presence of a wide range of bioactive peptides in jejunal effluents of humans fed milk proteins. The question arises about the role of these peptides in the intestinal tract where they may modulate physiologic functions. Additional studies performed on a complex food matrix, such as milk, should be helpful to understand how the interaction between proteins affect hydrolysis kinetics.

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The authors' responsibilities were as follows—CG and JL: designed the research; CG, GA, R Benamouzig, and R Boutrou: conducted the research; R Boutrou, AM-B, and JJ: analyzed data; R Boutrou, CG, DT, JL, and DD: wrote the manuscript; and R Boutrou and JL: had primary responsibility for the final content of the manuscript. None of the authors had a conflict of interest.

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