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Release of β-casomorphins 5 and 7 during simulated gastro-intestinal digestion of bovine β-casein variants and milk-based infant formulas

Ivano De Noni *

Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università Degli Studi di Milano, Via G. Celoria 2, Milano 20133, Italy

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ABSTRACT

The release of β-casomorphin-5 (BCM5) and β-casomorphin-7 (BCM7) was investigated during simulated gastro-intestinal digestion (SGID) of bovine β-casein variants ($n = 3$), commercial milk-based infant formulas ($n = 6$) and experimental infant formulas ($n = 3$). SGID included pepsin digestion at pH 2.0, 3.0 and 4.0 and further hydrolysis with Corolase PP™. β-Casein (β-CN) variants were extracted from raw milks coming from cows of Holstein-Friesian and Jersey breeds. Genomic DNA was isolated from milk and the β-CN genotype was determined by a PCR-based method. Phenotype at protein level was determined by capillary zone electrophoresis in order to ascertain the level of gene expression. Recognition and quantification of BCMs involved HPLC coupled to tandem MS. Regardless of the pH, BCM7 generated from variants A1 and B of β-CN (5–176 mmol/mol casein) the highest amount being released during SGID of form B. As expected, the peptide was not released from variant A2 at any steps of SGID. BCM5 was not formed in hydrolysates irrespective of either the genetic variant or the pH value during SGID. Variants A1, A2 and B of β-CN were present in all the commercial infant formulae (IFs) submitted to SGID. Accordingly, 16–297 nmol BCM7 were released from 800 ml IF, i.e. the daily recommended intake for infant. Industrial indirect-UHT treatments (156 °C × 6–9 s) did not modify release of BCM7 and, during SGID, comparable peptide amounts formed in raw formulation and final heat-treated IFs.

1. Introduction

Bovine milk proteins represent a source of biologically active peptides which may have "pharmacological" activity. These peptides are encrypted in inactive form within the sequences of milk protein from which they are released during either *in vivo* or *in vitro* digestion. Many peptides derived from bovine milk protein have been studied and widely reviewed in the literature (Meisel, 2001; Shah, 2000; Silva & Malcata, 2005; Tirelli, De Noni, & Resmini, 1997). β-Casomorphins (BCMs) are opioid-like peptides deriving from β-casein (β-CN) and were originally isolated from an enzymatic casein digest (Brantl, Teschemacher, Henschen, & Lottspeich, 1979). Among them, the most studied were BCM7 and BCM5 which represent fragments f60–66 and f60–64 of β-CN, respectively (Schlimme, Meisel, & Frister, 1989). They contain the common N-terminal amino acid sequence Tyr-Pro-Phe-Pro and possess preferential μ-receptor agonist activity (Brantl, Pfeiffer, Herz, Henschen, & Lottspeich, 1982; Sun & Cade, 1999).

Several PCR-based methods are available for analysing DNA of cows producing milk with different β-CN genotypes (Barroso, Dunner, & Ca ón, 1999; Medrano & Sharrow, 1991). In this regard, a PCR method using a microarray technology has been validated recently (Chessa et al., 2007). Although many genetic variants of β-CN have been recognized, A1 and A2 are the most widespread forms in dairy cattle breeds, especially in Holstein-Friesian (Farrell et al., 2004). The B variant is less common and it has been mostly recovered in Jersey breed. Unlike A1 and B variants, the presence of proline at position 67 in the primary sequence of A2 form would not allow the enzymatic cleavage of the molecule yielding BCM7 during gastro-intestinal digestion (Hartwig, Teschemacher, Lehmann, Gauly, & Erhardt, 1997; Jinsmaa & Yoshikawa, 1999). The formation and fate of bioactive peptides from milk proteins have been mainly investigated adopting protocols based on simulated gastro-intestinal digestion (SGID). Usefulness and reliability of these protocols depend upon both used enzymes and physiological factors related to the digestion processes in infants, such as the rate of gastric emptying and changes in gastric pH value. This last affects pepsin activity and therefore the rate and degree of protein degradation (Chatterton, Rasmussen, Heegaard, Sorensen, & Petersen, 2004).

Infant formulas (IFs) are designed to narrowly match the content and the performance of breast milk. Most of IFs are based on cows' milk or on hydrolysates of cows' milk protein when hypoallergenic formulas are considered (Caffarelli et al., 2002). Extensive research shows that human milk and breastfeeding of infants

* Tel.: +39 02 50316680; fax: +39 02 50316672.
E-mail address: ivano.denoni@unimi.it

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provide advantages with regard to general health, growth and development, while significantly decreasing the risk of acute and chronic diseases (Anonymous, 2005). The theory that excessive amounts of endogenous or exogenous opioid peptides may have pathophysiological relevance gained much scientific interest and the role of food opioid peptides has been deeply investigated in infants due to the fact that newborns have increased gut permeability and immature central nervous systems (Dettmer, Hanna, Whetstone, Hansen, & Hammock, 2007; Reichtel & Knivsberg, 2003). In this regard, BCMs have been reported to be involved in autism, diabetes, sudden infant death syndrome, asthma and hypertension (Elliott, Harris, Hill, Bibby, & Wasmuth, 1999; Sun & Cade, 1999; Sun et al., 2003). Although still disputed (Dairy reporter, 2007; Truswell, 2005), these findings attracted the interest of some dairy industries and drinking milk from cows with A2 β-CN genotype has been marketed in New Zealand and Australia since 2003 (A2 Corporation, www.a2corporation.com).

The release of BCMs from either bovine β-CN or IFs submitted to SGID including peptic attack at pH values close to that of infant's stomach has not been studied to date. Scant information is available about the amount of BCMs 5 and 7 that could form during SGID of IFs. Moreover, little is known about the effect of heating of milk on the release of these peptides. This work is aimed at investigating the occurrence and the level of BCM5 and BCM7 in hydrolysates of either alternative variants of bovine β-CN or commercial IFs submitted to SGID. The effect of heat load during manufacturing of IFs was also studied with respect to BCMs release during SGID. Due to difficulties in identification of BCMs in milk protein hydrolysates, HPLC and tandem mass spectrometry (MS/MS) were used for separation and quantification of these bioactive peptides.

2. Materials and methods

2.1. Pure BCMs and enzymes

Pure BCM5 (Tyr-Pro-Phe-Pro-Gly) and BCM7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) were purchased from Bachem (Bubendorf, Switzerland). Pepsin (P 7000) was from Sigma (St. Louis, MO, USA). Corolase PP™ was from Röhm (Darmstadt, Germany) and it represents a proteolytic enzyme preparation from pig pancreas that contains, in addition to trypsin and chymotrypsin, numerous amino- and carboxipeptidase activities.

2.2. Samples

Raw bulk milk and individual milks ($n = 4$) from either Holstein-Friesian or Jersey cows were used for β-CN extraction. Milk samples were cooled to 4–6 °C and promptly submitted to genotyping of β-CN polymorphism or stored at −24 °C until analysis.

Six cow-milk based IFs were purchased on the Italian market. IF-1, IF-2, IF-3 and IF-4 were powders whereas IF-5 and IF-6 were liquid formulas. Some characteristics of IFs are shown in Table 1. The IFs were suited for the normal full-term newborn infants during the first six months of life. Solutions of IF-1, IF-2, IF-3 and IF-4 were prepared by dissolving the powder using distilled water, according to the manufacturer's instructions. IF-5 and IF-6 were used as they were purchased.

Experimental liquid formulas IF-7, IF-8 and IF-9 were prepared from the industrial formulations whose composition is given in Table 2. IF-7 and IF-8 were heat treated at 152 °C/6 s and 152 °C/9 s, respectively, while IF-9 was UHT treated at 152 °C/6 s. An industrial indirect-UHT equipment (Alfa Laval, Monza, Italy) was used. IF-7 and IF-8 were suited for infants during the first six months of life, IF-9 was meant for infants 6–12 months aged. Both experimental IFs and unheated formulations were digested and analyzed.

2.3. β-casein polymorphism genotyping

Polymorphism genotyping was assessed according to Chessa et al. (2007) using a PCR method including a microarray technology approach based on ligation detection reaction (LDR) and an universal array (UA). A commercial kit (GFX Genomic Blood DNA Purification kit, Amersham Biosciences, Piscataway, NJ) was used for the DNA extraction directly from milk. The extraction was performed on 300 μl of fresh milk to recover at least 5 ng/μl of genomic DNA in a final volume of 100 μl. The amplification reaction was performed in a 50 μl mixture containing 15 μl of DNA solution (at least 75 ng), 1× AccuPrime PCR Buffer II, 1 μl of AccuPrime *Taq* DNA Polymerase (Invitrogen, Milano, Italy), 10–16 pmol of different primers (sequences are detailed by Chessa et al., 2007). The following conditions were used for the PCR assay: an initial denaturation step of 94 °C for 5 min was followed by 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 68 °C for 60 s, and a final extension step at 68 °C for 7 min using a PTC-200 DNA engine thermal cycler (MJ Research Inc., Waltham, MA). The DNA solution containing the PCR products was then purified by GFX PCR DNA and Gel Band Purification kits (Amersham Pharmacia Biotech Inc., Piscataway, NJ), eluted in 40 μl of autoclaved water and quantified using a Bioanalyzer 2100, and a DNA 7500 kit for 100–7500-bp dsDNA sizing and quantification (Agilent Technologies, Milano, Italy). Polymorphism genotyping was assessed using microarray technology approach based on the LDR-UA detailed by Chessa et al. (2007).

2.4. Capillary zone electrophoresis of raw milk samples

CZE was used to ascertain the level of gene expression and for evaluating the purity of β-CN variants extracted from raw milks. To this purpose, the method proposed by Recio and Olieman (1996) was adopted and a Beckman P/ACE 5500 (Beckman Coulter Inc., Fullerton, CA) equipment was used.

2.5. Extraction of β-casein

Extraction of β-CN was performed according to Huppertz et al. (2006) with some modifications. This procedure takes advantage of the hydrophobic characteristics of β-CN which allow this protein to escape casein micelles and to solubilize into whey at low temperature. Moreover, the procedure allows the recovery of protein

Table 1
Milk ingredients and protein content of commercial infant formulas submitted to SGID

Sample	Composition	Protein content (%, w/w)
IF-1	Milk whey and skim milk	11.3
IF-2	Skim milk and milk whey	12.5
IF-3	Skim milk and milk whey	9.5
IF-4	Skim milk and milk whey	11.0
IF-5	Skim milk and milk whey	1.2
IF-6	Skim milk and milk whey	1.3

Table 2
Main composition (%) and casein-to-whey protein ratio (w/w) of starting formulations used for manufacturing experimental infant formulas IF-7, IF-8 and IF-9

	IF-7 and IF-8	IF-9
Protein	1.5	1.3
Lactose	6.2	5.8
Maltodextrins	2.0	1.4
Fat	3.5	3.7
Casein:whey	70:30	50:50

without structural or chemical modifications. Aliquots of 500 ml raw milk with alternative β-CN variant, as determined by PCR-LDR-UA, were warmed to 30 °C. Reconstituted (25 mg l^{-1}) calf rennet powder (1:150.000, 96% chymosin) from Clerici (Cadorago, Italy) was added in order to achieve milk coagulation in about 15 min. The coagulum was cut and, after centrifugation at 5000*g* for 15 min at 30 °C, the whey was taken off. A volume of demineralised water (70 °C), equal to that of the drained whey, was added to the curd and the mixture was held at 70 °C for 5 min. Following centrifugation at 5000*g* for 15 min at 5 °C, the supernatant was discarded. The curd was finely cut, suspended in 500 ml demineralised water and left at 5 °C for 24 h. After centrifugation at 5000*g* for 15 min at 5 °C, the supernatant was filtered through filter paper and freeze dried. The purity of extracted β-CN was assessed by CZE of freeze-dried supernatants. Determination of casein-to-total nitrogen ratio was determined by the Kjeldahl method (IDF-International Dairy Federation, 1986).

Non-CN nitrogen was measured in the supernatant obtained after acidification to pH 4.6. CN-nitrogen was obtained by subtracting non-CN nitrogen from total nitrogen. Percentage of β-CN on total CN-nitrogen was based on peak areas resulting form CZE of CNs.

Prior to SGID, extracted β-CNs were dissolved (24 mg ml^{-1}) in UF permeate obtained by ultrafiltration of skimmed pasteurized milk through a hydrophilic 3000 Da cut-off membrane (Amicon, Beverly, MA).

2.6. Simulated gastro-intestinal digestion (SGID)

Digestions of either β-CN solutions or IFs were carried out according to the protocol proposed by Schmidt, Meijer, Slangen, and Van Beresteijn (1995). Samples were initially hydrolyzed by pepsin at pH 2.0, 3.0 or 4.0 (1:50 E/S ratio). Aliquots were taken out of the reaction mixture after 30 and 90 min. Pepsin inactivation was achieved by raising the pH to 7–8 with 1 N NaOH. The remaining part of the hydrolysis mixture was brought to pH 7.5 with 4 N NaOH and Corolase PP™ was added at 1:25 E/S ratio. Aliquots were taken out after 10, 30, 60 and 150 min and the enzyme was inactivated by heating (95 °C for 10 min). Aliquots were stored at −24 °C until HPLC/MS/MS analysis. Each sample was submitted to four replicate digestions on the same day and the mean amount of released BCMs was reported.

2.7. HPLC–MS/MS analysis

Separation and identification of BCM5 and BCM7 in hydrolysates from SGID were performed on an HPLC–MS apparatus. This consisted of an Alliance 2695 HPLC system (Waters, Milford, MA) combined with a Waters 996 diode array detector, and coupled to a LCQ Deca XP mass spectrometer with an electrospray interface (Thermo, San Jose, CA). The HPLC flow was split post-detector through a micro splitter valve (Upchurch Scientific, Oak Harbor, WA) to give a flow rate of 30 μl min^{-1} which was directed into the mass spectrometer *via* the electrospray interface.

Eluting solvents A and B were water/acetonitrile 97/5 (v/v) and water/acetonitrile 5/95 (v/v), respectively, both containing 0.1% (v/v) trifluoroacetic acid (Fluka, Buchs, Switzerland). Water and acetonitrile were from Fluka and Merck (Darmstadt, Germany), respectively. The elution gradient, as solvent B proportion, was as follows: 0–5 min, 0%; 5–65 min, 55%; 65–70 min, 100%; 70–72 min, 100%, 72–76 min, 0%. Flow rate was 0.2 ml min^{-1} and detection was carried out at 210 nm. A PLRP-S column (2.1 mm i.d. × 250 mm, 5 μm, 300 Å) from Polymer Laboratories (Church Stretton, Shropshire, UK) was used and kept at 40 °C.

The MS parameters were as follows: spray voltage 5000 V, sheath gas 25 (arbitrary units), aux gas 5 (arbitrary units), capillary temperature 300 °C, capillary voltage 32 V. Recognition of BCM5 and BCM7 in the hydrolysates was carried out by means of MS/MS of monoprotonated ions (i.e. *m/z* 580.3 for BCM5 and *m/z* 790.3 for BCM7) following these conditions: collision energy 35%, activation *q* 0.17 (BCM5) or 0.20 (BCM7). Calculation of BCMs amount was carried out by summing up the intensities of y_3 and b_3 ions or y_4 and y_5 ions for BCM5 and BCM7, respectively. To this purpose, 1–50 pmoles synthetic BCM5 or BCM7 were dissolved in water, submitted to HPLC/MS/MS and used as external standard for quantification. Triplicate HPLC–MS/MS analyses of the same digest showed good repeatability (CV < 5). Data were processed using Excalibur™ software (Thermo).

3. Results

3.1. Release of BCM5 and BCM7 during simulated gastro-intestinal digestion of different variants of β-casein

To investigate the effects of gastro-intestinal digestion on the release of BCM5 and BCM7, β-CNs of alternative genotype were incubated with pepsin and Corolase™. Initially, three homozygote (A1, A2 and B) and one heterozygote (A1/A2) raw milk samples were selected by PCR-LDR-UA. CZE of A1/A2 milk permitted to ascertaining the level of gene expression at protein level. Indeed, variant A2 was largely over-expressed with respect to the A1 form (Fig. 1). PCR showed variants A1, A2 and B to be present in the bulk milk sample and quantitative ratios among forms was established by means of CZE (Fig. 1). The effectiveness of the procedure for β-CN extraction was assessed by comparing the CZE patterns of the original raw milks to those of the derived extracts. In heterozygote samples, similar protein recovery was achieved for the different β-CN variants (not shown). In all extracts, β-CN represented 40–60% of total protein.

β-CN-enriched extracts were dissolved in UF milk permeate and submitted to peptic attack at pH 2.0 or at pH 3.0 and 4.0 in order better to simulate gastric digestion in infant's stomach. Usage of permeate was aimed at performing digestion in a *medium* as close to milk as possible. Formation of peptides was followed by HPLC and, as shown in Fig. 2 for aliquots taken during SGID variant A1, most of the peptides were released after addition of Corolase PP™. Similar chromatographic patterns were obtained for digests of A2 and B variants of β-CN (not shown). Due to the presence of peptides eluting at retention times close to those of pure BCM5 (29.1 min) and BCM7 (37.3 min), recognition of BCM5 and BCM7 was based on HPLC–MS/MS of the monoprotonated ($[M+H^+]^{+1}$) precursor ions of these peptides (i.e. *m/z* 580.3 for BCM5 and *m/z*

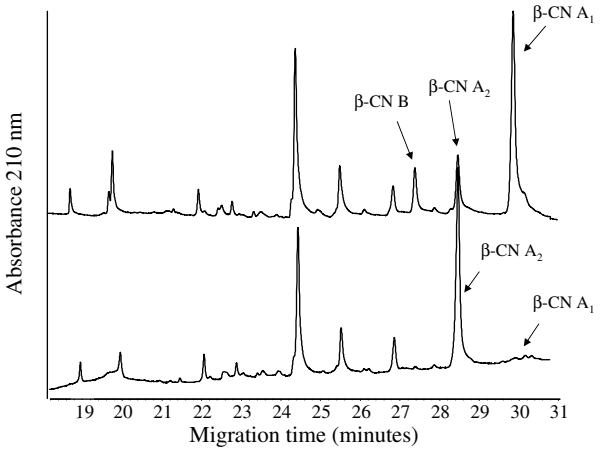

Fig. 1. CZE patterns of the heterozygote milk samples containing A1/A2 and A1/A2/B forms of β-CN.

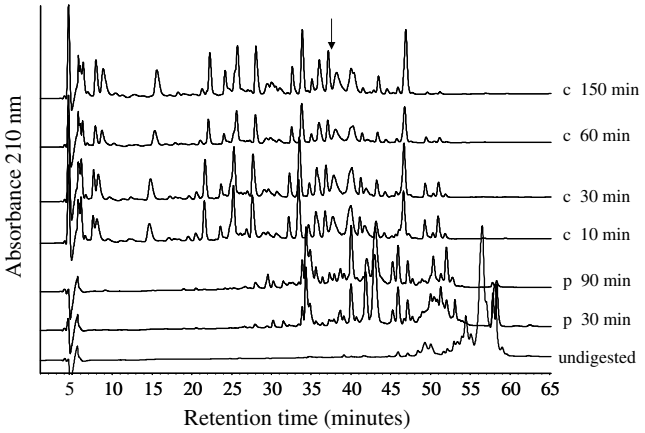

Fig. 2. HPLC/UV chromatograms of aliquots taken at different steps during SGID of extract from milk sample of A1/A2/B genotype. Abbreviations indicate sampling time (minutes) after (p) pepsin or (c) Corolase PP™ addition. Arrow indicates retention time (37.3 min) of BCM7.

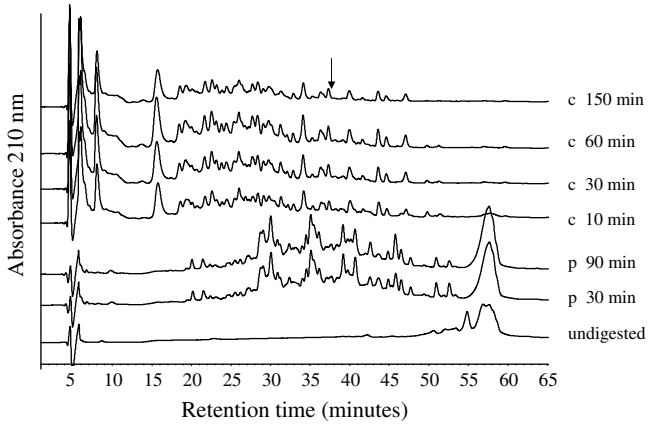

Fig. 3. HPLC/UV chromatograms of aliquots taken at different steps during SGID of reconstituted commercial IF-1. Abbreviations indicate sampling time (minutes) after pepsin (p) pepsin or (c) Corolase PP™ addition. Arrow indicates retention time (37.3 min) of BCM7.

790.3 for BCM7). The fragmentation spectrum of BCM7 resulted in formation of very abundant y-ions since these ions are normally over-represented in MS/MS spectra of proline-rich peptides. The sum of the intensities of the two major y-ions at *m/z* 383.1 and 530.2, representing y_4 and y_5 ions, respectively, was used for quantification of BCM7 in digests. As expected, BCM7 was not released during initial peptic digestion of extracts regardless of both the pH value and the β-CN genotype (not shown). Even after digestion with Corolase PP™, BCM7 was not released from variant A2 of β-CN (Table 3). Variants A1 and B released 7–54 mmol BCM7/mol β-CN and 85–176 mmol BCM7/mol β-CN, respectively, being the highest peptide amount recovered in the digest of variant B when peptic attack at pH 2.0 was included (Table 3). After SGID, 5–16 mmol BCM7/mol β-CN were formed in the digest of extract containing A1/A2/B forms (Table 3). During Corolase PP™ attack, BCM7 accumulated in all digests (not shown) and its stability to further degradation was confirmed by SGID of synthetic BCM7 (not shown). Hydrolysis of extract containing A1/A2 variants resulted in the release of 0.5 and 0.2 mmol BCM7/mol β-CN at pH 3.0 and 4.0, respectively (Table 3). As previously described, the CZE patterns of the heterozygote A1/A2 milk and the derived extract showed the presence of a small peak assignable to variant A1 of β-CN (Fig. 1). Scant presence of this form likely accounted for the negligible release of BCM7 during SGID. With the exception of B variant the highest amounts of BCM7 formed during SGID including hydrolysis with pepsin at pH 4.0 (Table 3). Contrary to what was observed for BCM7, BCM5 was not recovered at any steps of SGID regardless of either pH values or β-CN variant.

Table 3
Release of BCM7 from different variants of β-CN submitted to SGID including pepsin hydrolysis at different pH values

Variant	mmol BCM7/mol β-CN[a]	% of theoretical maximum BCM7
A1 (pH 2)	20 ± 6	1.9
A1 (pH 3)	7 ± 1	0.7
A1 (pH 4)	54 ± 12	5.4
A2 (pH 2)	0.0	0
A2 (pH 3)	0.0	0
A2 (pH 4)	0.0	0
B (pH 2)	176 ± 33	17.6
B (pH 3)	85 ± 13	8.5
B (pH 4)	102 ± 18	10.2
A1/A2 (pH 2)	0.0	0
A1/A2 (pH 3)	0.5 ± 0.07	0.05
A1/A2 (pH 4)	0.2 ± 0.04	0.02
A1/A2/B (pH 2)	12 ± 3	1.2
A1/A2/B (pH 3)	5 ± 1	0.5
A1/A2/B (pH 4)	16 ± 3	0.2

[a] Mean value of 4 replicated digestions ± standard error.

3.2. Release of BCM5 and BCM7 during simulated gastro-intestinal digestion of infant formulas

All the six commercial IFs contained A1, A2 and B forms of β-CN. As an example, Fig. 3 shows the HPLC/UV chromatograms of aliquots taken during SGID of reconstituted IF-1. As found for β-CN-enriched extracts, extensive hydrolysis occurred during protein digestion with Corolase PP™ and about 40–50 peaks were present in the HPLC/UV chromatogram at the end of SGID. Consequently, only HPLC–MS/MS was adopted for recognition and quantification of BCM5 and BCM7 in digests (Fig. 4). No BCM5 was released during SGID whereas BCM7 was formed during Corolase PP™ attack in digests of all samples at concentrations corresponding to 16–297 nmol/800 ml reconstituted IF (Table 4). This volume represents the recommended daily intake as indicated on the labels of the studied commercial IFs. As expected, the lowest BCM7 amounts (18–38 nmol/800 ml) formed in IF-1 which contains whey protein as the main dairy ingredient (Table 1).

Experimental IF-7 and IF-8 were from a starting formulation containing casein and whey protein at 70:30 ratio that was then UHT-treated at 152 °C/6 s and 152 °C/9 s, respectively. As consequence of SGID, the starting formulation released 102–174 nmol BCM7/800 ml (Table 5). Similar amounts were found in digests of IF-7 (109–169 nmol BCM7/800 ml) and IF-8 (94–142 nmol BCM7/800 ml). When the formulation contained casein and whey protein at 50:50 ratio (IF-9), 54–124 nmol and 46–128 nmol BCM7 were found in 800 ml of the unheated formulation and of IF-9, respectively. As expected, less BCM7 released from IF-9 with respect to IF-7 and IF-8 because of the lower CN content of the former.

4. Discussion

Unlike A1 and B variants, the A2 form of β-CN has proline at position 67 that would not allow the enzymatic cleavage of the β-CN molecule yielding BCM7. Data from literature concerning BCM7 release have been derived from enzymatic digestions which included initial peptic attack of β-CN at pH 2.0. However, the pH optimum for pepsin is far from the pH values (3–5) typical of infant's stomach (Chatterton et al., 2004). The paramount role of pepsin on

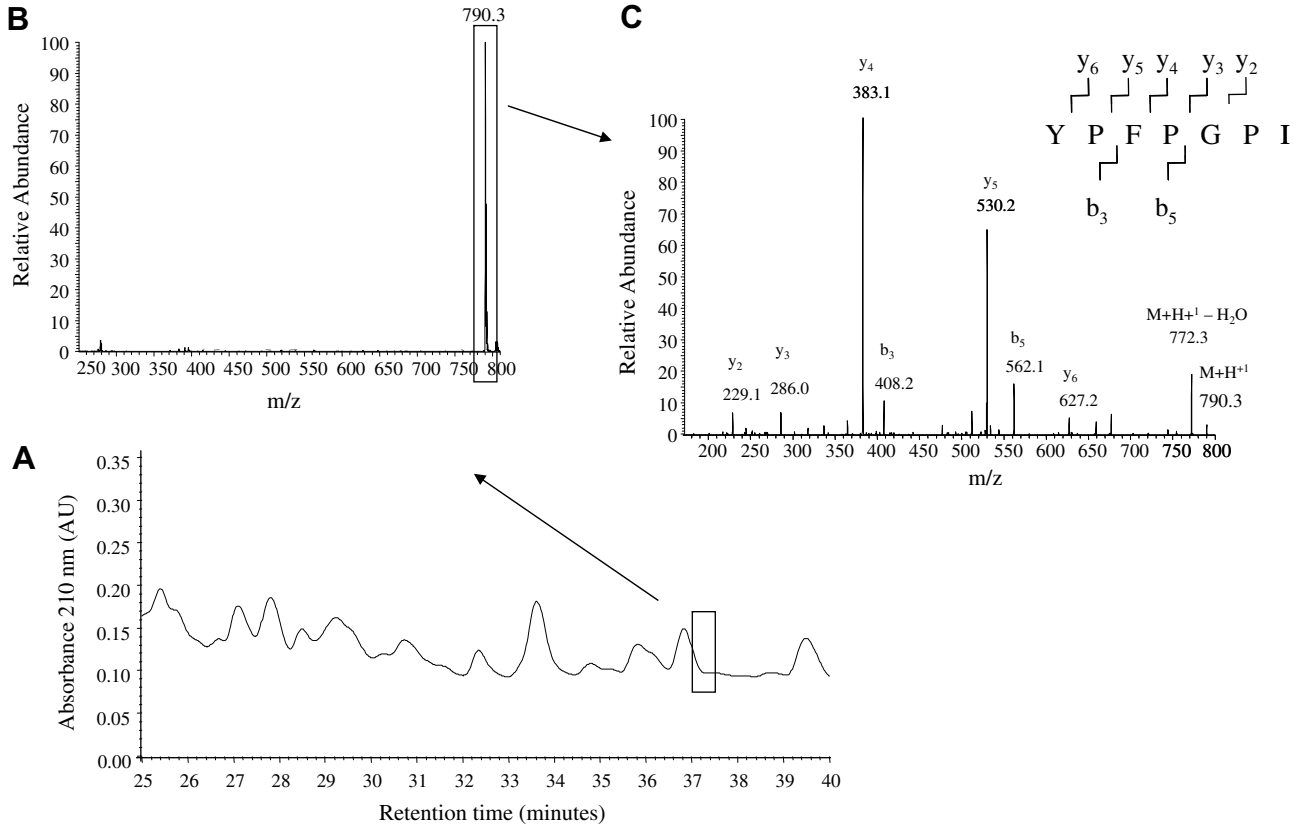

Fig. 4. (A) HPLC/UV chromatogram of reconstituted commercial IF-1 at the end of SGID (25–40 min of pattern "c 150 min" in Fig. 3). (B) Mass spectrum of the selected chromatographic area in (A). (C) Tandem mass spectrum of ion at *m/z* 790.3. The sequence of BCM7 is displayed with the fragment ions observed in the spectrum. For clarity, only b- and y-type ions are labeled.

Table 4
Release of BCM7 from commercial infant formulas submitted to SGID including pepsin hydrolysis at different pH values

Sample	nmol BCM7/800ml[a]
IF-1 (pH 2)	38 ± 6
IF-1 (pH 3)	33 ± 4
IF-1 (pH 4)	18 ± 4
IF-2 (pH 2)	212 ± 36
IF-2 (pH 3)	297 ± 41
IF-2 (pH 4)	156 ± 18
IF-3 (pH 2)	61 ± 9
IF-3 (pH 3)	66 ± 7
IF-3 (pH 4)	16 ± 3
IF-4 (pH 2)	122 ± 20
IF-4 (pH 3)	72 ± 14
IF-4 (pH 4)	67 ± 7
IF-5 (pH 2)	180 ± 31
IF-5 (pH 3)	95 ± 13
IF-5 (pH 4)	118 ± 18
IF-6 (pH 2)	130 ± 19
IF-6 (pH 3)	202 ± 21
IF-6 (pH 4)	271 ± 47

[a] Mean value of 4 replicated digestions ± standard error.

Table 5
Release of BCM7 from starting formulation (SF) and related heat-treated IFs during SGID including pepsin hydrolysis at different pH values

Sample	nmol BCM7/800 ml[a]
SF (pH 2)	165 ± 14
SF (pH 3)	102 ± 10
SF (pH 4)	174 ± 23
IF-7 (pH 2)	109 ± 11
IF-7 (pH 3)	166 ± 13
IF-7 (pH 4)	169 ± 8
IF-8 (pH 2)	94 ± 10
IF-8 (pH 3)	121 ± 12
IF-8 (pH 4)	142 ± 17
SF (pH 2)	64 ± 9
SF (pH 3)	54 ± 7
SF (pH 4)	124 ± 15
IF-9 (pH 2)	46 ± 6
IF-9 (pH 3)	128 ± 10
IF-9 (pH 4)	124 ± 13

[a] Mean value of 4 replicated digestions ± standard error.

BCM7 release has been investigated by Jinsmaa and Yoshikawa (1999). These authors found only negligible levels of BCM7 in the elastase-leucine aminopeptidase digest of β-CN when the preliminary pepsin treatment at pH 2.0 was omitted. To date, no study has ascertained whether a pepsin attack at pH > 2 can affect BCM7 release during SGID of β-CN and IFs. In the present work, SGID was carried out in the pH range 2–4 so taking into account the gastric pH values reported for infants. The amount of BCM7 (7–54 mmol/mol β-CN) generated from A1 variant overlapped that (17 mmol BCM7/mol β-CN) found in a pepsin-elastase-leucine amino peptidase hydrolysate of β-CN by Jinsmaa and Yoshikawa (1999). In their work, β-CN was isolated from milk of a Holstein-Friesian cow and, therefore, A1 and A2 were the variants likely submitted to digestion. Although, a clear effect of pH value during pepsin attack was not indicated in our work, the highest amounts of BCM7 formed at pH 4.0 with the exception of variant B (Table 3). The availability of long peptides as substrates for intestinal proteases

could account for this effect. At pH > 2, pepsin action is depressed and more and longer intact peptides are expected to reach the intestine (Chatterton et al., 2004). These peptides are generally either BCMs 9, 13 and 21 or (Val_{59})-BCMs (Jinsmaa & Yoshikawa, 1999). They generate from initial cleavage of the Leu_{58}-Val_{59} peptide bonds by pepsin followed by further degradation by intestinal proteases. Recognition of these peptides was not performed in digests prepared in the present work, while (Val_{59})-BCM9 was recently recovered in a IF after digestion with pepsin (pH 3.5) and porcine pancreatin (Hernandez-Ledesma, Quirós, Amigo, & Recio, 2007).

As expected, BCM7 was not released from variant A2 at any step of SGID irrespective of the pH value adopted for pepsin attack. These findings confirmed data from literature which came from enzymatic hydrolysis including pepsin attack at pH 2.0 only (Hartwig et al., 1997; Jinsmaa & Yoshikawa, 1999). In a recent work, a homozygote A2 milk was reported to release BCM7 after digestion with pepsin (Cieslinska, Kaminski, Kostyra, & Sienkiewicz-Szlapka, 2007). The recovered amount of BCM7 was four times lower than that released from a homozygote A1 milk. These findings disagree with both data from literature and our own results. Nevertheless, the same findings appear unreliable since they resulted from both a 24-h peptic digestion (pH 2.0) and recognition/quantification of BCM7 based on HPLC/UV. As shown in Fig. 2, HPLC/UV patterns of milk protein digests comprise many co-eluting peptides and quantification of individual components is reliable only if specific approaches, such as tandem MS or peptide sequencing, are adopted.

Negligible amounts of BCM7 (0.2–0.5 mmol BCM7/mol β-CN) were released during SGID of extract from heterozygote milk containing A1/A2 variants. This fact related to the presence of low amount of A1 form in the extract (Fig. 1). Different concentration of individual CNs in milk can be due to the variability in levels of gene expression that characterises variants of CNs containing changes in non coding regions (Bleck, Conroy, & Wheeler, 1996). These variants proved to influence protein synthesis through either decreased mRNA stability or different transcription rate (Rando et al., 1998). Contrary to BCM7, BCM5 was not formed regardless of either the digested variant or the pH value adopted for peptic attack. This finding confirmed previous results of Svedberg, De Haas, Leimenstoll, Paul, and Teschemacher (1985). In their work, considerable amount of BCM7 but not BCM5 was found in *in vitro* digests of bovine milk. In our work, simultaneous release and degradation of BCM5 was excluded since, as found for BCM7, pure BCM5 proved to be stable during SGID (not shown).

As far as IFs are concerned, no BCM5 formed after SGID of the six commercial IFs considered here. On the contrary, levels in the range 16–297 nmol BCM7/800 ml were found (Table 4). Recently, BCM7 was recovered (but not quantified) in UF permeate of a reconstituted IF submitted to peptic attack at pH 3.5 and further digestion with Corolase PP™ (Hernandez-Ledesma, Amigo, Ramos, & Recio, 2004). In another work, the same authors did not report presence of this peptide in a IF digested with pepsin (pH 3.5) and porcine pancreatin (Hernandez-Ledesma et al., 2007). If the mere presence of A2 variant in this IF is not assumed, some questions raise about the interchangeability of the currently adopted protocols for *in vitro* digestions. This especially applies when the release of peptides with high proline content has to be studied. For this reason, a proteolytic enzyme preparation including several amino- and carboxipeptidase activities, in addition to trypsin and chymotrypsin, was used in the present work.

Despite the supposed food safety issues deriving from consumption of A1 and B variants of β-CN (Dairy Reporter.com, 2007; Truswell, 2005), BCM7 is reported to be involved in etiology of several diseases (Elliott et al., 1999; Sun et al., 2003). According to Elliott et al. (1999), incidence of Type I diabetes in children correlates with consumption of milk with β-CN A1 genotype and even more with A1 + B variants. In the present work, it is worth noting that the highest amount of BCM7 was released from variant B regardless of the pH value adopted for peptic attack during SGID (Table 3). Little information is available in literature concerning the minimum amount of BCM7 capable to exert physiological effects *in vivo* or *ex vivo*. According to Jarmolowska, Kostyra, Krawczuk, and Kostyra (1999), 0.05% BCM7 would be sufficient to cause intestinal contractions. Much lower amounts (200–4000-fold) of BCM7 would be present in infant's gut after digestion of 800 ml of the commercial IFs considered here (Table 4). On this basis, this biological activity would be excluded. Exploitation of more severe pathophysiological effects implies absorption of BCM7 through gastro-intestinal mucosa, resistance to cleavage during blood transportation and ability to cross brain barrier (Reid & Hubbel, 1994). Given these considerations, the established levels of BCM7 occurring in the studied milk-based IFs would not be problematic.

Severe heat treatment of starting formulation in manufacturing of IFs guarantees safety of the final product but it can also induce glycation and crosslinking of milk protein (Henle, 2005). In this regard, little information is available on digestibility of CNs in heated food with respect to rate and degree of bioactive peptide release. According to Chatterton et al. (2004), heating of bovine CNs during processing of IFs slightly increased susceptibility to proteolysis. In our work, industrial indirect-UHT treatments did not modify release of BCM7 and, during SGID, comparable peptide amounts formed in starting formulation and in final heat-treated IFs (Table 5). Despite this, further research is needed to ascertain the effects exerted by different or additional treatments on BCMs release.

5. Conclusion

The present results confirm the release of BCM7 during SGID of variants A1 and B of bovine β-CN. They also demonstrate that release from variant A2 does not occur also when pepsin digestion is carried out at a pH peculiar of infant's stomach. Irrespective of the ongoing debate on β-CN healthiness, data from SGID of IFs bring additional insight into the field of infant nutrition and provide further information for the evaluation of the potential bioactivity of bovine milk protein used in the manufacturing of IFs.

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