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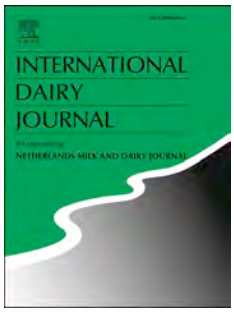

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Identification of bioactive peptides and quantification of β-casomorphin-7 from bovine β-casein A1, A2 and I after ex vivo gastrointestinal digestion

Tora Asledottir[a], Thao T. Le[b], Bjørn Petrat-Melin[b], Tove G. Devold[a], Lotte B. Larsen[b], Gerd E. Vegarud[a]

[a] *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Chr. Magnus Falsens vei 1, 1430 Aas, Norway*

[b] *Department of Food Science, Aarhus University, Blichers Allé 20, 8830 Tjele, Denmark*

Corresponding author. Tel.: +47 91581582

E-mail address: tora.asledottir@nmbu.no (T. Asledottir)

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ABSTRACT

This study investigated whether different genetic variants of β-CN give rise to different bioactive peptides during digestion. β-CN was purified from bovine milk of genetic variants A1, A2 and I, and digested with human gastrointestinal juices in a static ex vivo model. Mass spectrometry analyses revealed that the peptide ^{60}YPFPGPIPN^{68} was exclusively identified from variants containing proline at position 67. Most strikingly, the opioid peptide β-casomorphin-7, ^{60}YPFPGPI^{66}, was identified from both variants A1 and A2 after simulated digestion, though with concentration being somewhat higher after digestion of the variant A1, compared with variants A2 and I. The peptides ^{134}HLPLP^{138} and ^{133}LHLPLP^{138} were both identified after initial 5 min of duodenal digestion. In conclusion, genetic variation of β-CN may affect proteolysis during digestion; however, the release of BCM7 does not seem to be linked solely to variant A1, as earlier suggested by relevant published literature on in vitro digestion.

1. Introduction

Caseins (CNs) are the most abundant proteins in ruminant milk and are known to be an important source of bioactive peptides (BAP). The β-CN constitutes about 40% of total CN in bovine milk, and is encoded by the *CSN2* gene on chromosome 6. This gene is highly polymorphic and to date, fifteen different genetic variants of β-CN have been identified (Caroli, Chessa, & Erhardt, 2009; Gallinat et al., 2013). The A1 genetic variant of β-CN contains the amino acid histidine at position 67 of the mature β-CN sequence, whereas the variants A2 and I contain proline at this position. Enzymatic release of peptides during digestion may be affected by the genetic variant of the protein due to the enzymes' affinity for certain amino acids (AA). Furthermore, certain BAPs released during digestion can interact with receptors on target cells in the human body and induce physiological responses including antioxidant, antihypertensive, opioid, antimicrobial and immunomodulatory (Nongonierma & FitzGerald, 2015).

β-Casomorphin-7 (BCM7) is a BAP encrypted in the mature β-CN sequence, which can be released through enzymatic hydrolysis during digestion. A number of studies have described the release of BCM7 after A1 β-CN ingestion (Barnett, McNabb, Roy, Woodford, & Clarke, 2014; Boutrou et al., 2013; De Noni, 2008; Jinsmaa & Yoshikawa, 1999; Ul Haq, Kapila, & Kapila, 2015). It has also been hypothesised, but not yet confirmed, that BCM7 can lead to the development of non-communicable diseases such as cancer, cardiovascular diseases and autistic disorders. Atherosclerosis development has been detected in a rabbit model after digestion of A1 β-CN (Tailford, Berry, Thomas, & Campbell, 2003). Current reported studies have centred on potential links between A1 and A2 β-CN variants and digestion effects, especially in lactose intolerants or people reporting some unspecific kind of encountered milk intolerance (Jianqin et al., 2016). In addition, a correlation between

diabetes incidence and consumption of A1 β-CN has been suggested in a study comparing national cow milk consumption and the frequency of childhood diabetes in several countries (Elliott, Harris, Hill, Bibby, & Wasmuth, 1999). However, the study by Elliott et al. (1999) was an ecological study and did not account for several confounding factors.

In 2009 the European Food Safety Authority (EFSA) published a review on all relevant scientific literature concerning A1 β-CN intake (EFSA, 2009). EFSA concluded that the studies evaluated in the review were not of sufficient quality, where the human intervention studies were limited due to small number of subjects and short intervention periods. In addition, some health effects associated with BCM7 absorption had not been reproducible. Consequently, EFSA could not establish a cause-effect relationship between the oral intake of BCM7 or related peptides, and the development of any suggested non-communicable diseases. More comprehensive studies were required to eliminate other factors that could be responsible for outcomes in previous studies, and a formal risk assessment of food-derived peptides was not recommended. Although, conversely, another review has recently pointed at some potential relationships between A1 milk ingestion and milk intolerance experience through BCM7 (Pal, Woodford, Kukuljan, & Ho, 2015).

In vitro model digestion is generally performed with single enzymes from porcine or bovine origin. These commercial enzymes provide an easy alternative of studying digestion and it has recently been established a consensus model for human in vitro digestion (Minekus et al., 2014). However, human gastrointestinal (GI) juices contain a mixture of different enzymes, inhibitors and salts, and this may be a more realistic mimic when considering the environment of the human digestion. This method has therefore been referred to as an ex vivo approach, as the enzymes used are aspirated from human volunteers, and used ‘as they are’ in an in vitro environment.

Consequently, the aim of our study was to investigate the degradation profile of

purified β-CN genetic variants A1, A2 and I after ex vivo GI digestion, with particular interest in the release of BCM7. The results are discussed to evaluate whether AA substitutions in the β-CN protein sequence are responsible for different cleavage sites during digestion, and, moreover, to assess whether the use of human GI enzymes better represents in vivo digestion than commercial enzymes of non-human origin.

2. Materials and methods

2.1. Isolation and purification of β-casein

Skimmed milk samples were collected from individual Danish Holstein cows (one cow per milk sample). The cows were homozygous for the β-CN variants A1, A2 and I as determined by liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI/MS) analysis of retention times and molecular masses of β-CN variants present in the milk (Jensen et al., 2012) and purified by adaptation of purification method earlier published (Petrat-Melin et al., 2015). In brief, the frozen milk samples were thawed at 4 °C for 48 h with stirring for the last 24 h. The samples were then ultracentrifuged (150,000 × *g* at 4 °C for 2 h) and β-CN was isoelectrically precipitated from the supernatants. To recover the purified β-CN from the solution, the samples were washed three times with Milli-Q following centrifugation (1000 × *g* at 4 °C for 10 min), before being lyophilised.

2.2. Identification of β-casein variants by liquid chromatography-electrospray ionisation-mass spectrometry

Lyophilised β-CN fractions were analysed to confirm their identity and purity. The method was based on previous studies by Jensen et al. (2012) and Petrat-Melin et al. (2015). Briefly, ten milligram of lyophilised β-CN fractions were dissolved in 1 mL of a solution containing 6 M guanidine hydrochloride (GndHCl) and 100 mM bis-Tris. Fresh dithioerythritol (DTE) was added to a final concentration of 15 mM. Samples were filtered through a 0.2 μm polytetrafluoroethylene filter (Mini-Uniprep, Whatman, GE Healthcare Life Sciences, New Jersey, US). The samples were analysed using high performance liquid chromatography (HPLC) on an HPLC 1100 system (Agilent Technologies, Santa Clara, CA), with a Jupiter C4 column (250 × 2 mm, 5 μm particle size, 300 Å pores; Phenomenex, US) operated at 40 °C, coupled to an ESI single-quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) for identification of milk proteins. All β-CN samples were analysed in duplicate. Integrated peak areas from the chromatogram with absorption at 214 nm were used to calculate the relative protein content in the purified samples.

2.3. Determination of protein purity of isolated β-casein variants

The protein concentration of the purified β-CN fractions was determined by UV absorption using the molecular extinction coefficients, as previously described by Petrat-Melin et al. (2015). One milligram of lyophilised β-CN fractions were dissolved in 1 mL of 6 M GndHCl and 100 mM bis-Tris. The absorbance was measured in a Cary 60 UV/Vis spectrophotometer (Agilent Technologies, US) at 280 nm. The measured absorbance was used to calculate the purity of the variants together with the predicted absorbance of 1 mg mL^{-1} protein. The calculation was based on earlier work by Edelhoch (1967), that described the determination of molecular extinction coefficients at 280 nm (ε280) of W, Y and C. The samples were analysed in quadruplicate.

2.4. Human gastrointestinal juices

Human gastric and duodenal juices were collected according to Ulleberg et al. (2011) and approved by the Regional Committees for Medical and Health Research Ethics (REC) in Norway. Aspiration of 20 healthy volunteers from age 20 to 42 was performed at Moss Hospital, Norway. The volunteers were fasting for at least 8 h prior to aspiration. The gastric and duodenal juices were aspirated simultaneously through a three-lumen silicon tube, and the aspirates were stored at –20 °C, then at –80 °C until further use. The pepsin and trypsin activities of the human GI juices were assayed according to COST Action INFOGEST protocol (Minekus et al., 2014) prior to the simulated digestion.

2.5. Ex vivo gastrointestinal digestion of β-casein variants

Ex vivo GI digestion was preformed according to the INFOGEST protocol (Minekus et al., 2014), with some modifications. Commercial enzymes were substituted with human GI juices and the oral phase was omitted, as the digestion of diluted β-CN solution requires neither chewing nor addition of amylase. The experiments were carried out in 50 mL tubes containing 10 mg mL^{-1} β-CN diluted in simulated milk ultrafiltrate (SMUF; Jenness & Koops, 1962). The samples were incubated in a water bath at 37 °C with magnetic stirring. Sampling was done after 60 min in the gastric phase and after 5 and 120 min in the duodenal phase. Enzyme inactivation at sampling point was done by increasing the pH above 6 with 1 M $NaHCO_3$ in the gastric samples, and by adding 5 mM Pefabloc® (Sigma Aldrich, St. Louis, USA) to the duodenal samples. The digestion was performed in two independent experiments and all samples were immediately frozen and kept at –20 °C.

2.6. Protein hydrolysis profile by sodium dodecyl sulphate polyacrylamide gel electrophoresis

The undigested and digested β-CN was mixed (1:1) with fresh sodium dodecyl sulphate (SDS) buffer containing 5 mM dithiothreitol (DTT) and heated at 95 °C for 5 min. Ten microlitres of sample was loaded in each well of a 10% Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK), and run at 200 V for 25 min. Low molecular mass protein ladder was used as a standard. The proteins were fixed in 20% methanol and stained in Comassie Brilliant Blue (Bio-Rad Laboratories Ltd), then de-stained and kept in preservation solution (10% glycerol and 10 % methanol). Images were captured by Gel Doc EZ Imager (Bio-Rad Laboratories Ltd).

2.7. Peptide identification of digested β-casein by liquid chromatography-electrospray ionisation-tandem mass spectrometry ion trap

The peptide analyses were performed with one of the two independent experiments from GI digestion. Samples were diluted in ammonium bicarbonate (0.05 M) to an initial CN concentration of 1 mg mL^{-1}, before being reduced (by DTE, at 60 °C for 10 min) and alkylated (by iodoacetamide, at 37 °C for 30 min in dark). Formic acid (6.25 %) was added to acidify the samples. The solution was filtered through a 10 kDa molecular mass cut-off spin-filter (Millipore, Cork, Ireland) by centrifugation at 14,000 × *g* and at 4 °C for 10 min. All samples were diluted 3-fold in 0.1% formic acid prior to LC-ESI-tandem MS analysis.

The digests were analysed on an Aeris Peptide C18 column of dimensions 250 mm × 2.1 mm, with a particle size of 3.6 μm (Phenomenex, Torrance, CA, USA) of an Agilent LC

1200 series operated at 40 °C, and directly connected to an HCT Ultra Ion Trap (Bruker Daltonics, DE, USA). A linear LC gradient, consisting of solvent A (0.1 % formic acid) and B (90% acetronitrile, 0.1% formic acid), was set as: 0–40% B over 80 min and increasing to 80% B over 15 min, with flow rate at 200 µL min^{-1}. Sample injection volume was 5 µL. The mass scan for MS mode was from 250 to 1800 *m/z* and MS/MS mode was from 100 to 1800 *m/z*. DataAnalysis (version 4.0) and Biotools (version 3.1) (Bruker Daltonic) were used to process the MS/MS spectra. The data was then sent to Mascot (Matrix Science, MA, USA) and searched against a custom in-house database with search parameters as follows: *Bos Taurus*, none for proteolytic enzyme, and no modifications for identification of peptides. Peptide hits above the Mascot score significant threshold ($P < 0.05$) were accepted, and peptides of interest below threshold were manually analysed.

2.8. Quantification of β-casomorphin-7 by multiple reaction monitoring mass spectrometry

The digests were analysed on a 1260 Infinity LC system (Agilent Technologies, Waldbronn, DE, USA) coupled to a 6460 Triple Quad (QQQ) mass spectrometer (Agilent Technologies). BCM7 was separated on a C18 column (2.1 mm × 50 mm, 1.8 µm, Agilent Technologies) at 45 °C. The mobile phases contained (A) 0.1% formic acid in Milli-Q water and (B) 90% acetonitrile in Milli-Q water with 0.1% formic acid at a flow rate of 550 µL min^{-1}. The gradient was as follows: 0–40% B over 18 min and increasing to 80% B over 5 min. The injection volume was 10 µL. The 6460 QQQ was operated in multiple reaction monitoring (MRM) mode with dwell time of 200 ms and fragmentor of 165 V. A list of Q1/Q3 masses of BCM7 (790.2/383.1 and 790.2/530.0) was submitted as a batch for data acquisition. The optimal collision energy (CE) was 30 eV. Serial standard concentration was

25, 50, 75, 125 and 250 fmol μL^{-1}. Isotope labelled (C^{13} and N^{15} at F) internal standard with the concentration of 50 fmol μL^{-1} was spiked into the standard solutions and the digested samples. Standard peptide BCM7 and isotope labelled BCM7 was purchased from ThermoFisher Scientific (Biopolymers, Ulm, Germany) as AQUA Ultimate. Purity of these peptides was above 97%. Quantification was calculated based on ratio of the analyte and internal standard by MassHunter Quantitative Analysis software (Agilent Technologies). The MRM method was validated for linearity, repeatability, limit of detection (LOD) and limit of quantification (LOQ). The samples were measured in quadruplicates.

3. Results and discussion

3.1. Purification of the β-casein variants

β-Casein from homozygous milk was isolated for the genetic variants A1, A2 and I. Chromatograms from LC-ESI/MS provided the molecular mass of the proteins in the purified samples, which enabled the identification of the β-CN variants by confirming the homozygosity and genotype with masses of 24,018 Da, 23,978 Da and 23,960 Da for variants A1, A2 and I, respectively (Fig. 1). Considering the relative amount of protein in milk, the yield from purification of β-CN was calculated by the amount (mg) retrieved from the milk samples, and from the electropherograms obtained from capillary electrophoresis of each milk sample (data not shown). The yield was estimated to be between 16% (A1) and 30% (I), which is expected due to the process of cold storage, where only the β-CN loosely bound to the other CNs through hydrophobic interactions and released into the whey fraction by the cooling procedure was obtained.

The purity of β-CN relative to total protein in the isolates was determined by LC-ESI-

MS analysis and predicted absorbance at 280 nm, and varied from 90% to 93% (Table 1). These data are comparable with previously reported results by Petrat-Melin et al. (2015), who reported purity of ≈ 90% with the same method for purification. This method is useful as it combines both predicted and measured absorbance, which gives a better estimation of the absolute protein content. Purification of β-CN by cold storage and ultracentrifugation is simple and limits the risk of changing the physiochemical properties of the proteins, as could occur with urea-based methods (O'Mahony & Fox, 2013; Petrat-Melin, 2014).

3.2. Digestion of β-CN

Purified β-CN was digested according to the INFOGEST protocol (Minekus et al., 2014), with some modifications due to the use of human enzymes. The human digestive juices comprise various enzyme isoforms, and this may broaden their spectrum of cleavage (Ulleberg et al., 2011). Subsequently, the enzyme activities in the human juices were calculated on the basis of the ratio between substrate and enzyme secretion (v/v) in an "in house" protocol. Eriksen et al. (2010) conducted a study on digestion with porcine and human GI enzymes and concluded that the human juices perhaps should be preferred over pure commercial enzymes when simulating human digestion, as they observed differences in the degradation pattern of the proteins by human juices compared with commercial enzymes.

A preliminary study of the digested proteins by sodium docecylsulphate-polyacrylamide gel-electrophoresis (SDS-PAGE) was done to ensure that the enzyme activity was sufficient for hydrolysis of proteins and compared with earlier data from Islam, Ekeberg, Rukke, and Vegarud (2015). The degradation profiles illustrated in Fig. 2 proved that the time chosen in the gastric and duodenal phases were sufficient for enzymatic digestion of β-CN. Large variation in gastric transit times of 15 min to 3 h have been recorded, depending

on the texture and viscosity of the food bolus (Guerra et al., 2012), and the intestinal phase can last up to 5 h. Furthermore, the INFOGEST protocol states that 2 h in each phase is sufficient for digestion, as it represents half-emptying time of a moderately nutritious and semi-solid meal (Minekus et al., 2014). However, in the study performed by Islam et al. (2015), CNs showed almost complete degradation after 40 min in the gastric phase with human gastric juice, and the remaining proteins (whey) were degraded after 5 min in the duodenal phase with human duodenal juice. These results laid the basis for the chosen digestion time in our study.

As illustrated in Fig. 2, SDS-PAGE did not reveal any obvious, visual differences in the degradation pattern among the variants. After initial 60 min of gastric digestion, the major fraction of β-CN was digested and only small faint bands were visible around < 20 kDa. The visible bands around 30–60 kDa (a and b) represented the human duodenal enzymes, reported by Devle et al. (2014). It is expected that the genetic variants can produce different peptides, however, these peptides may be of low concentration and smaller than 10 kDa, and is therefore not visible in the gel. Some faint bands around 10–20 kDa in the gel were visible after 60 min of gastric digestion. These bands may represent degraded β-CN, or it could represent traces of whey proteins that were detected in the isolated β-CN after purification.

3.3. Peptide identification by mass spectrometry

Peptides formed during digestion of β-CN were characterised by LC-ESI/MS/MS and submitted to a custom in-house Mascot database. All peptides identified with significant hits ($P < 0.05$) from the Mascot database search, or manually analysed spectra, are illustrated in Fig. 3, with their respective position in the β-CN AA sequence. In total, 109 different peptides were identified with 75% sequence coverage, where the N-terminal of the protein

had fewer identified stretches. The coverage is in consistence with a previous study reported by Schmelzer, Schöps, Ulbrich-Hofmann, Neubert, and Raith (2004), who identified 41 peptides with 75% sequence coverage after simulated gastric digestion of β-CN. In a later study, Schmelzer et al. (2007) identified 125 peptides with 100% sequence coverage. However, the digestion was performed solely in the gastric phase with porcine pepsin and the peptide identification was performed by both matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) and LC-ESI/MS/MS. Schmelzer et al. (2004) also identified fewer peptides in the N-terminal region of the protein. A possible explanation could be the phosphorylation of serine residues in this region, and poor ionisation of phosphopeptides in the ESI/MS source, which can lead to low detection of peptides unless phosphorylation enrichment is applied. Nor was the phosphorylation taken into account when specifying the Mascot search for known peptides. In other words, peptide fractions with phosphorylation might not have been identified.

Furthermore, during the gastric phase of digestion, the active enzyme pepsin hydrolyses the proteins. This enzyme cleaves preferably at sites of F, L and Y (Rawlings, Barrett, & Finn, 2016); however, pepsin can cleave with a more complex action as well, depending on the AA combination upstream or downstream of the cleavage site (Tang, 1963). Several sites of cleavage were observed after digestion, including at residue P and the average length of all identified peptides decreased from thirteen AA in the gastric digestion to an average length of nine AA at the end of duodenal digestion. P is considered rather resistant to proteolytic cleavage, thus the complex mixture of different isoforms of GI human juices may have enhanced the diverse cleavage.

After 60 min gastric digestion, the peptide ^{81}PVVVPPFLQPEVL^{93} with the M^{93} to L^{93} substitution was identified from the genetic variant I. Similar peptides were identified from variant A1 and A2, however, containing M^{93}. There were no other distinctive differences in

peptides identified among the genetic variants from the gastric digestion. The most frequent peptides exclusively observed after duodenal digestion were peptides derived from region Q^{56} to V^{82} in the mature β-CN sequence, with more diverse cleavage sites at residues T, Q, L, V and P. There were some distinct differences between the genetic variants and peptides found in this region. Four peptides cleaved N-terminal of position 67 belonging to variant A1 were identified, containing the P^{67} to H^{67} substitution. However, several peptides containing I^{66} at the C-terminal were identified among all variants. This indicates that the hydrolysis by human duodenal juices at position 67 is not only dependent on the residue H^{67}. Furthermore, peptides from the region H^{67} to V^{82} of variant A2 and I were missing in the peptide profile, but fragments downstream of this cleavage site were identified. This could be a result of non-identified peptides, rather than non-existing peptides.

Gastrointestinal digestion of β-CN resulted in identification of peptides previously described as bioactive, and is presented in Table 4. In the gastric phase of digestion two BAPs were identified: the ACE-inhibitory peptide ^{193}YQEPVLGPVR^{202} was identified from variants A1 and A2, and the antimicrobial and immunomodulatory peptide ^{193}YQEPVLGPVRGPFPIIV^{209} was identified from all three variants. In the duodenal phase of digestion the ACE-inhibitory peptide ^{6}LNVPGEIVE^{14}, previously reported by Gobbetti, Stepaniak, De Angelis, Corsetti, and Di Cagno (2002), was also identified from all variants. However, one peptide exclusively identified from the A2 variant after 120 min duodenal digestion was ^{60}YPFPGPIPN^{68}. This is a BAP reported by Saito, Nakamura, Kitazawa, Kawai, and Itoh (2000), where the residues I^{66} and P^{67} at the C-terminus of the peptide showed potent ACE-inhibitory effect. However, the author reported that this peptide had rather low anti-hypertensivity, which is explained by the large size of the molecule, that requires further digestion by intestinal enzymes before absorption. Moreover, the ACE-inhibitory and anti-oxidative peptide ^{59}VYPFPGPIPN^{68} was observed after duodenal

digestion of both variant A2 and I. This peptide is also referred to as V-BCM9, and showed to increase the IC_{50} value almost 22-fold, caused by the addition of V at the N-terminus of the BCM-peptide (Eisele, Stressler, Kranz, & Fischer, 2013). Another study performed by Petrat-Melin, Le, Møller, Larsen, and Young (2017) found that the A1 variant of this same peptide had a 5-fold decrease in IC_{50} value, making the peptide a stronger ACE inhibitory agent than the A2 variant of V-BCM9.

The ACE-inhibitory peptide ^{133}LHLPLP^{138}, previously reported by Quirós et al. (2007), was identified from both variant A1 and A2. This peptide has shown to be resistant to gastrointestinal digestion, but hydrolysed to its active form ^{134}HLPLP^{138} by brush border peptidases in the intestinal epithelium prior to absorption (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). However, in our study this pentapeptide was identified already after 5 min digestion in the duodenal phase, which also indicates the broad activity of the human digestive juices. Moreover, two derived peptide fragments from hydrolysis of the aforementioned hexapeptide have also previously been detected in plasma; i.e., the tetrapeptide fragments ^{134}HLPL^{137} and ^{135}LPLP^{138} were detected 5 min after oral administration of HLPLP in rats (Sánchez-Rivera et al., 2014). In addition, the tripeptide ^{134}HLP^{136} was detected with a rising concentration of the peptide until 60 min of incubation in plasma (Sánchez-Rivera et al., 2016).

Furthermore, peptides that showed homology for known AA sequence but were lacking the identity, where manually analysed by identification of b and y ions in MASCOT. The peptide illustrated in Fig. 4 is ^{60}YPFPGPI^{66} identified from variant A1 after 5 min of duodenal digestion. The characterisation was based on the LC-ESI-MS/MS of the double charged $[M+2H]^{2+}$ precursor ion with *m/z* 395.8, as well as singly charged BCM7. The opioid peptide BCM7 appeared after 120 min for variant A2 and I, compared with variant A1 where it was identified after initial 5 min. As explained earlier, the peptide bond N-terminal to

residue H^{67} is considered more prone to cleavage by proteolytic enzymes than P^{67}; hence, the cleavage may occur at a higher rate when H is present. However, duodenal protease activity increases with time, making sites of cleavage more diverse, and residues Q, S, P and N were also observed as effective cleavage sites, as previously reported by Petrat-Melin (2014). This suggests that the residue P^{67} of variant A2 and I may also affect the proteolytic cleavage.

In addition to the identification of BCM7, several BCM7-like peptides were identified after digestion of all three variants of purified β-CN. These peptides were cleaved at the C-terminal position of I^{66} and at the N-terminal position of Q^{56}, S^{57}, L^{58} or V^{59}. The identification of these peptides supports the theory of generation of BCM7 after digestion of both variant A1 and A2 β-CN. However, the N-terminal AA of BCM-like peptides is not the same as of BCM7. This may affect the absorption, transport and the binding of the peptides to opioid receptors (Nagpal et al., 2011). The release of BCM7 has been investigated in several studies after GI digestion of milk and milk-based products (Cieslinska, Kaminski, Kostyra, & Sienkiewicz-Szlapka, 2007; De Noni, 2008; Hernández-Ledesma, Amigo, Ramos, & Recio, 2004; Schmelzer et al., 2004; Ul Haq et al., 2015). In a study performed on different genetic variants of β-CN, release of BCM7 was observed from variant A1 and B (which also contains H^{67}) after simulated GI digestion (De Noni, 2008). The author did not observe release of BCM7 from variant A2. To our knowledge there are no data from literature that reveals the generation of BCM7 from A2 β-CN during simulated GI digestion with human enzymes.

3.4. Quantification of BCM7

BCM7 was quantified by multiple reaction monitoring (MRM) method. The singly charged peptide $YPFPGPI^{+1}$ was predominant and selected as precursor ion (Q1). The two

most abundant fragmentation ions y4 (*m/z* 383.1) and y5 (*m/z* 530.0) were chosen as product ions (Q3). There were two MRM transitions (Q1/Q3) for data acquisition. The absolute quantification was calculated based on a linear curve where the X axis was the concentration of peptide standard and the Y axis was the ratio of standard over internal standard. The standard calibration curve showed good linearity with R^2 values higher than 0.9995. LOD and LOQ values were very low at 0.01 and 0.04 fmol μL^{-1}, respectively.

The quantification method detected a significantly higher release of BCM7 during digestion of variant A1 than A2 and I ($P < 0.001$), and there were no significant differences in the release of the peptide between the variants A2 and I ($P > 0.05$). Four milligrams BCM7 per gram β-CN was detected from variant A1 after 120 min duodenal digestion, compared with approx. 1.4 mg from variant A2 and I (Fig. 5). This confirmed that the amount of BCM7 was higher when released from A1 β-CN, which was expected due to the identification of the peptide after initial 5 min duodenal digestion. However, the difference in the amount of BCM7 released from variant A1 or A2/I was relatively low, only a 2.8-fold increase in the amount when released from variant A1 containing H^{67}. Cieslinska et al. (2007) has also showed that hydrolysis of A2 β-CN by pepsin releases BCM7; however, the concentration of the peptide was four times higher when produced from the homozygous A1 β-CN. Moreover, the study by Cieslinska et al. (2007) was not performed under physiologically relevant conditions as the peptic digestion lasted for 24 h.

In a later study, Cieslinska et al. (2012) quantified BCM7 in hydrolysed milk of β-CN variant A1 and A2 and observed a 12-fold decrease in the concentration of BCM7 when released from A2. Our results of BCM7 (4 mg g^{-1}) are also higher than those of Jinsmaa and Yoshikawa (1999), who reported BCM7 yields of 0.57 mg g^{-1} after digestion of β-CN with pepsin, pancreatin and leucine aminopeptidase, and observed no release from β-CN with P^{67}. In addition, a recent cross-over clinical trial measured the plasma BCM7 concentration after

consumption of commercial milk, and observed no significant increase after the trial period with A1/A2 milk consumption compared with the washout period (Deth, Clarke, Ni, & Trivedi, 2016).

Other studies have also demonstrated BCM7 identification and quantification in milk and in several commercial cheeses (Nguyen, Johnson, Busetti, & Solah, 2015). Common production of cheese usually requires heat treatment of the milk as well as addition of rennet and starter culture, for pasteurisation, separation of whey and ripening. These processes can significantly affect the proteolysis of the cheese, subsequently releasing peptides during processing including BCMs. De Noni and Cattaneo (2010) observed the presence of BCM7 and BCM5 in several commercial cheeses, however, these cheeses were not produced from milk of homozygous variants, rather batched milk (as it occurs in the industries) containing the variants A1, A2 and B.

As discussed earlier, there is a diverse release of BAPs deriving from CN during GI digestion. In addition to the above-mentioned, other BAPs derived from CNs have been described to possess activities such as mineral binding, antithrombotic and to serve as opioid antagonists (Chabance et al., 1995; Silva & Malcata, 2005; Xu, 1998). The method and database used for identification of peptides (LC-ESI/MS/MS) in our study did not allow identification of peptides smaller than five AA long. Other methods need to be applied to identify BAPs such as the ACE-inhibitors VPP and IPP. These di- and tripeptides are often isobaric and co-eluting, which makes them difficult to separate and identify (Lahrichi, Affolter, Zolezzi, & Panchaud, 2013).

Despite the fact that BCM7 was detected after digestion of β-CN with human GI enzymes, it may not be absorbed intact in the intestines, and if it is absorbed, it may still be hydrolysed by brush border peptidases (e.g., dipeptidyl peptidase-4) before reaching its target organ (e.g., opioid receptors). However, Barnett et al. (2014) observed a delay in GI transit

417 time after A1 β-CN consumption relative to A2, and an increase in myeloperoxidase activity
418 in the colon and DPP-4 activity in the jejunum after A1 β-CN administration compared with
419 A2. This suggests that the opioid peptide BCM7 may lead to bloating, constipation or
420 diarrhoea prior to absorption. Moreover, other comprehensive methods need to be applied to
421 suggest if the peptide could be linked to the development of some non-communicable
422 diseases (e.g., permeability studies, plasma concentration measurements, in vivo studies).

423 Guerra et al. (2012) reviewed different approaches for simulating human digestion,
424 where static and dynamic models were discussed concerning their limitations and challenges.
425 The overall concluding remark was that it is impossible to fully mimic the digestive
426 parameters in vivo in a single simulated digestion model (in vitro/ex vivo). Digestive
427 processes such as hormonal and neural regulation, feedback mechanisms, mucosal cell
428 activity, peristaltic movements and the influence of the immune system are complex
429 parameters to fit into one model. Subsequently, combinatorial approaches have been
430 performed for the evaluation of intestinal permeability and the bioavailability of digested
431 compounds (Deat et al., 2009; Foltz et al., 2008; Osborne et al., 2014).

432 Moreover, the properties of epithelial cells in the small intestines have been studied
433 for designing models that exert the mechanisms of absorption. Caco-2 monolayers are widely
434 used as potent in vitro models to predict absorption of BAPs. Hydrolysis of BAPs by brush
435 border enzymes has been reported by Iwan et al. (2008), who demonstrated the effect of
436 dipeptidylpeptidase-4 (DPP4) on BCM7. The authors found that the presence of DPP4-
437 inhibitor increased transport of BCM7, and that DPP4 was the main factor of limiting the
438 half-life of opioid peptides. Osborne et al. (2014) reported similar results conducting peptide
439 permeability studies with Caco-2 cell monolayer, where rapid hydrolysis of BCM7 was
440 observed generating three peptide metabolites, YP, GPI and FPGPI. Furthermore, the degree
441 of the intestinal permeability is also an important factor, and individuals with leaky guts may

therefore be more prone to physiological effects of BAPs due to a higher permeability of the intestinal wall. De Magistris et al. (2010) found that the intestinal permeability was abnormal in children suffering from autistic spectrum disorders. However, in other studies evaluating food-derived opioids in urine output of children, there was no evidence linking autism to opioid peptides or to DPP4 deficiency (Cass et al., 2008; Hunter, O'Hare, Herron, Fisher, & Jones, 2003).

4. Conclusion

The use of human enzymes in simulation of human digestion has led the research one step forward towards in vivo digestion, with an ex vivo approach. The present study showed that different genetic variants of β-CN can affect the hydrolysis by gastrointestinal proteases, thus affecting peptides formed. It has also been established that ex vivo digestion of β-CNs leads to generation of several BAPs; however, the release of BCM7 is potentially not solely dependent on the genetic variants with residue H at position 67 in the AA sequence of β-CN. We have identified BCM7 from all variants, and the difference in the concentration between A1 and A2/I was rather low. These results imply that when studying release of BAPs during digestion, human enzymes should be preferred. Moreover, there is a need for evaluating the bioavailability of generated BAPs, as their function after release in the intestines is rather ambiguous. Irrespective of the on-going debate regarding health effects of BCM7 and other peptides, the data attained in this study brings additional clarification on the possible generation of BCM7, but the results still need to be confirmed by in vivo studies.

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646

Figure legends

Fig. 1. Chromatogram of purified β-CN variant A1, A2 and I. Each β-CN variant is illustrated with a peak at its retention time and corresponding mass (dalton, Da). Minor peaks represent the other CNs and whey proteins in the samples. All samples were analysed in duplicates (n = 2). CN, casein.

Fig. 2. Protein hydrolysis profile by SDS-PAGE of purified β-Casein, representative for all three variants after ex vivo digestion: STD, low molecular mass standard; 0, undigested; G60, gastric digestion with HGJ for 60 min; D5 and D120, duodenal digestion with HDJ for 5 and 120 min, respectively, after initial 60 min of gastric digestion; a and b, human duodenal enzymes; HGJ, human gastric juice; HDJ, human duodenal juice.

Fig. 3. Peptide fractions identified by tandem mass spectrometry after ex vivo digestion of purified β-casein with genetic variants A1, A2 and I. Peptides identified after 60 min of gastric digestion with HGJ are illustrated in red; peptides after 60 min of gastric digestion and 5 min of duodenal digestion with HGJ and HDJ are illustrated in green; peptides after 60 min of gastric digestion and 120 min of duodenal digestion with HGJ and HDJ are illustrated in blue. The marked region is peptides identified as BCM7 or BCM-like peptides. Arrows denote sites of amino acid substitution for different genetic variants; position 67 (A1) P→H; position 93 (I) M→L. HGJ, human gastric juice; HDJ, human duodenal juice.

Fig. 4. Representative chromatogram and spectra, here illustrated with variant A1. The same peptide (BCM7) was found from all variants in GI digestion. Panel A, base peak chromatogram for the mass 395.8 of purified β-CN after 60 min of gastric digestion and

5 min of duodenal digestion with human GI juices. Panel B, mass spectrum of marked peak in panel A. Panel C, tandem mass spectrum of ion with *m/z* 790.6 from panel B. Following sequence identification and MASCOT search, the MS/MS spectrum matches the β-CN sequence ^{60}YPFPGPI^{66} (BCM7). BCM7, β-casomorphin-7; CN, casein; GI, gastrointestinal.

Fig. 5. Concentration of BCM7 in digests of β-casein variants A1, A2 and I. Quantification was done by multiple reaction monitoring mass spectrometry after 60 min gastric digestion and 120 min duodenal digestion with human enzymes. Error bars with SEM (n = 4). **P*<0.001 compared with A2/I. BCM7, β-casomorphin-7.

Table 1

Mean relative protein content (%) of β-casein after purification of skimmed milk. [a]

β-CN genetic variant	β-CN	κ-CN	α_{S1}-CN	α_{S2}-CN	Whey proteins
A1	92.6 ± 0.17	1.3 ± 0.03	4.2 ± 0.20	1.5 ± 0.18	0.4 ± 0.22
A2	92.5 ± 0.27	0.8 ± 0.06	3.2 ± 0.22	1.7 ± 0.01	1.8 ± 0.41
I	90.2 ± 0.12	2.5 ± 0.08	5.0 ± 0.01	1.3 ± 0.01	1.0 ± 0.03

[a] Abbreviation: CN, casein. The purity of β-CN was calculated by peak areas from chromatograms obtained by LC-MS measured at 214 nm; values are expressed as mean percentage ± SD (n=2).

Table 2

Peptide fragments derived from β-casein (β-CN) with bioactivity (from literature), identified by tandem mass spectrometry (LC-ESI-MS/MS) after simulated digestion of purified β-CN variants A1, A2 and I, by human gastrointestinal juices. [a]

Phase of digestion	Digested β-CN variant	Position	Sequence	Bioactivity	Reference
Duodenal	A1, A2, I	6–14	LNVPGEIVE	ACE-inhibitor	Gobbetti et al. (2000)
Duodenal	A2, I	59–68	VYPFPGPIPN	ACE-inhibitor, antioxidative	Eisele et al. (2013)
Duodenal	A1, A2, I	60–66	YPFPGPI	Opioid	Brantl et al. (1981)
Duodenal	A2	60–68	YPFPGPIPN	ACE-inhibitor	Saito et al. (2000)
Duodenal	A1, A2	133–138	LHLPLP	Antihypertensive (in vivo)	Quirós et al. (2007)
Gastric	A1, A2	193–202	YQEPVLGPVR	ACE-inhibitor	Silva and Malcata (2005)
Gastric	A1, A2, I	193–209	YQEPVLGPVRGPFPIIV	Antimicrobial, immunomodulatory	Sandré et al. (2001)

[a] Position refers to position in the mature β-CN sequence; the amino acid sequence is given using one-letter abbreviations.

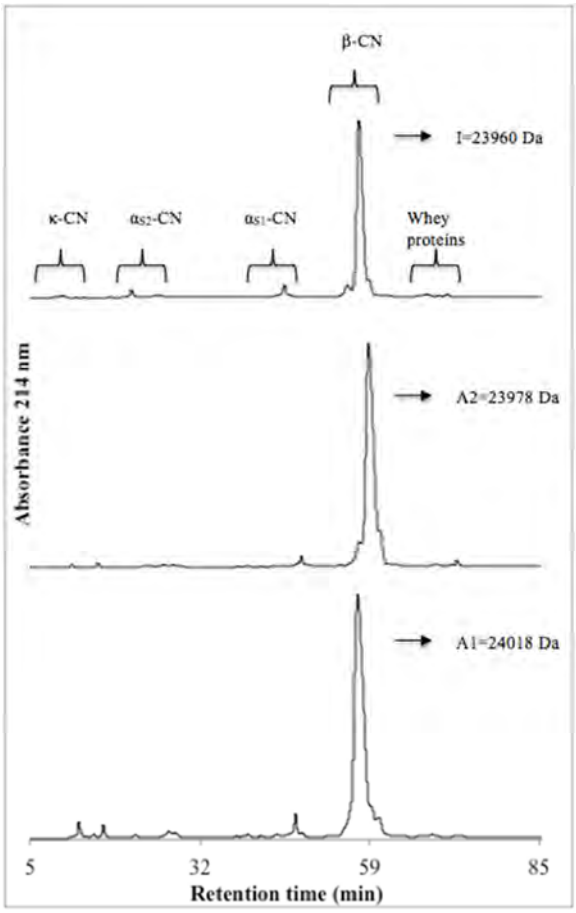

Figure 1

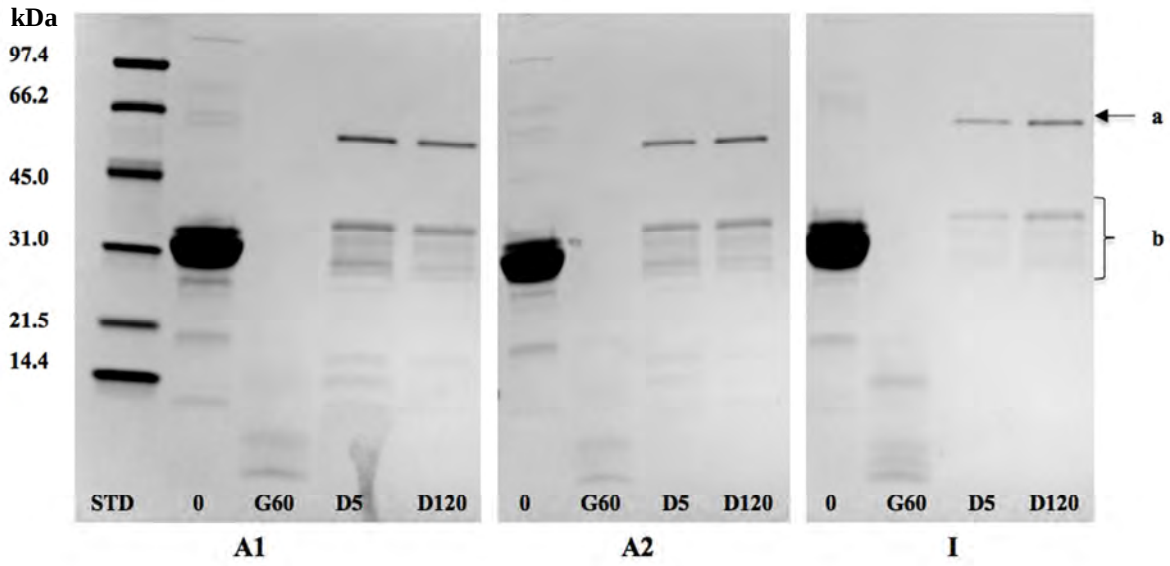

Figure 2.

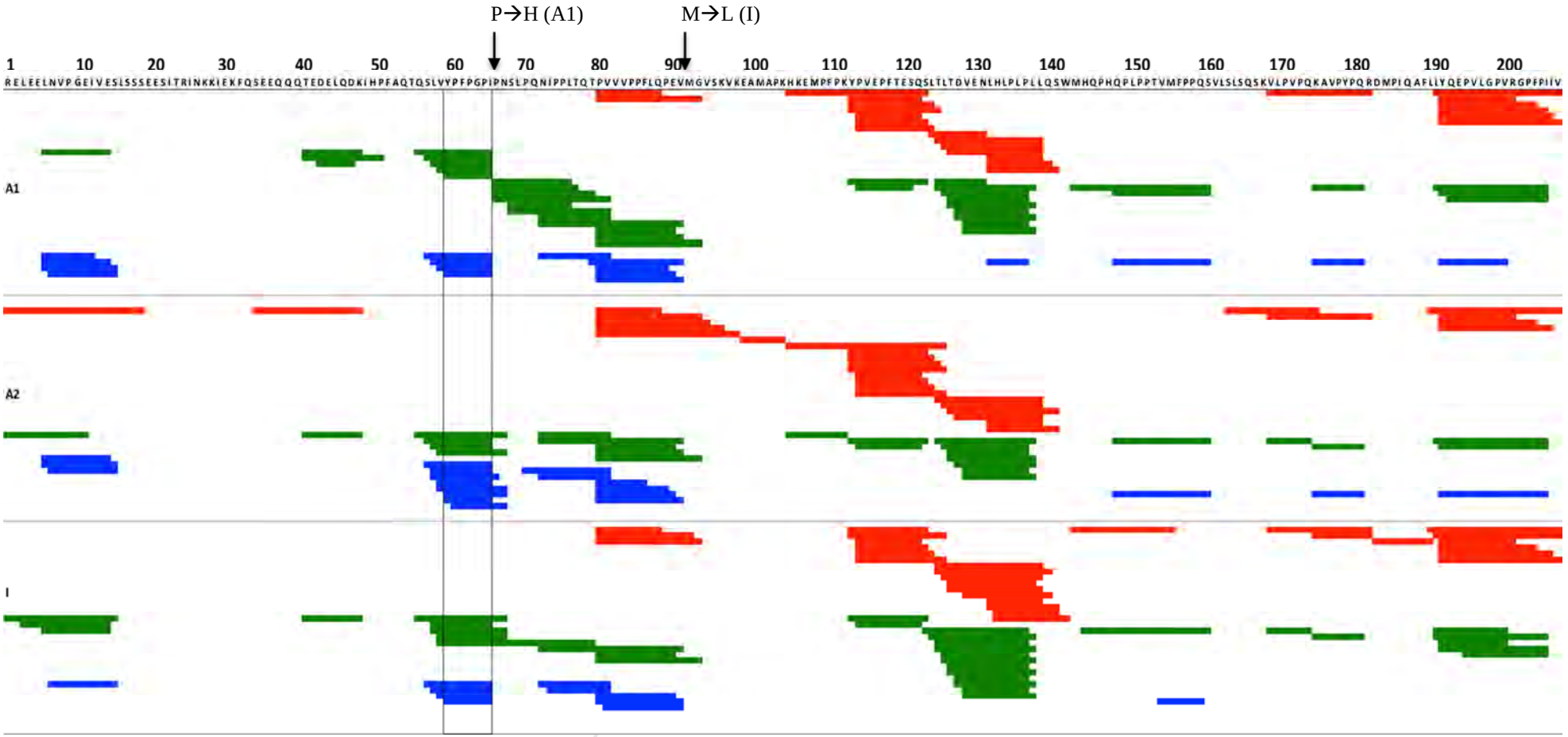
P→H (A1)
M→L (I)
1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200
RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
A1
A2
I

Figure 3

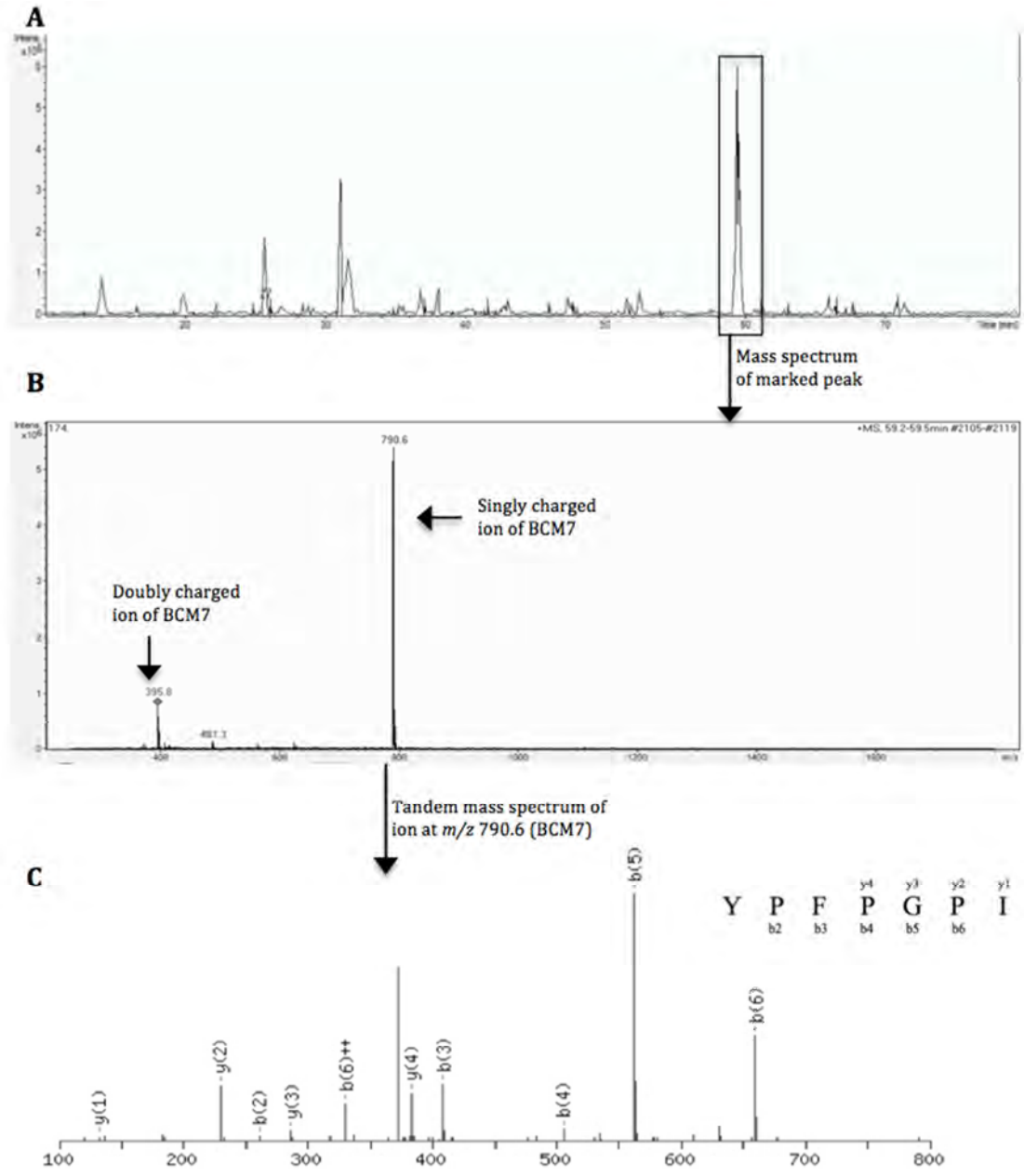
A
Mass spectrum
of marked peak
B
790.6
Singly charged
ion of BCM7
Doubly charged
ion of BCM7
395.8
Tandem mass spectrum of
ion at m/z 790.6 (BCM7)
C
Y P F P G P I
b2 b3 b4 b5 b6
y4 y3 y2 y1
b(5)
b(6)
y(2)
y(4)
b(3)
b(6)++
y(1)
b(2)
y(3)
b(4)
100 200 300 400 500 600 700 800

Figure 4.

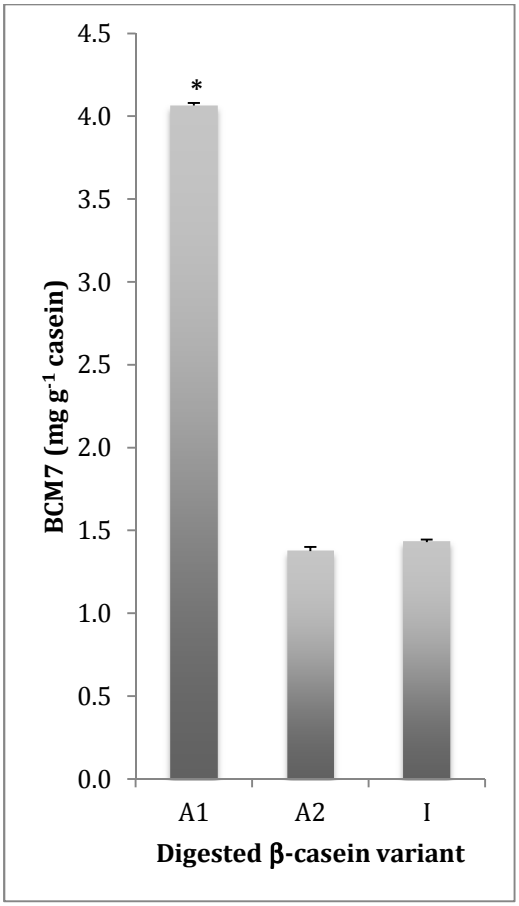

Figure 5.