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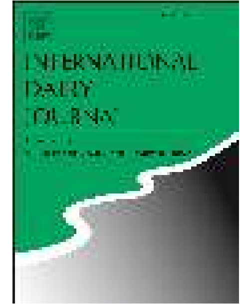

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PII: S0958-6946(17)30069-9

DOI: 10.1016/j.idairyj.2017.03.008

Reference: INDA 4159

To appear in: *International Dairy Journal*

Received Date: 23 December 2016

Revised Date: 13 March 2017

Accepted Date: 15 March 2017

Please cite this article as: Asledottir, T., Le, T.T., Petrat-Melin, B., Devold, T.G., Larsen, L.B., Vegarud, G.E., Identification of bioactive peptides and quantification of β-casomorphin-7 from bovine β-casein A1, A2 and I after ex vivo gastrointestinal digestion, *International Dairy Journal* (2017), doi: 10.1016/j.idairyj.2017.03.008.

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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

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3 ABSTRACT

4

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

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18 **1. Introduction**

19

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

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

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

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

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

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

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

73

74 **2. Materials and methods**

75

76 *2.1. Isolation and purification of β-casein*

77

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

88

89 *2.2. Identification of β-casein variants by liquid chromatography-electrospray ionisation-*
90 *mass spectrometry*

91

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92 Lyophilised β-CN fractions were analysed to confirm their identity and purity. The
93 method was based on previous studies by Jensen et al. (2012) and Petrat-Melin et al. (2015).
94 Briefly, ten milligram of lyophilised β-CN fractions were dissolved in 1 mL of a solution
95 containing 6 M guanidine hydrochloride (GndHCl) and 100 mM bis-Tris. Fresh
96 dithioerythritol (DTE) was added to a final concentration of 15 mM. Samples were filtered
97 through a 0.2 μm polytetrafluoroethylene filter (Mini-Uniprep, Whatman, GE Healthcare Life
98 Sciences, New Jersey, US). The samples were analysed using high performance liquid
99 chromatography (HPLC) on an HPLC 1100 system (Agilent Technologies, Santa Clara, CA),
100 with a Jupiter C4 column (250 × 2 mm, 5 μm particle size, 300 Å pores; Phenomenex, US)
101 operated at 40 °C, coupled to an ESI single-quadrupole mass spectrometer (Agilent
102 Technologies, Palo Alto, CA, USA) for identification of milk proteins. All β-CN samples
103 were analysed in duplicate. Integrated peak areas from the chromatogram with absorption at
104 214 nm were used to calculate the relative protein content in the purified samples.
105
106 *2.3. Determination of protein purity of isolated β-casein variants*
107
108 The protein concentration of the purified β-CN fractions was determined by UV
109 absorption using the molecular extinction coefficients, as previously described by Petrat-
110 Melin et al. (2015). One milligram of lyophilised β-CN fractions were dissolved in 1 mL of
111 6 M GndHCl and 100 mM bis-Tris. The absorbance was measured in a Cary 60 UV/Vis
112 spectrophotometer (Agilent Technologies, US) at 280 nm. The measured absorbance was
113 used to calculate the purity of the variants together with the predicted absorbance of 1 mg
114 mL^{-1} protein. The calculation was based on earlier work by Edelhoch (1967), that described
115 the determination of molecular extinction coefficients at 280 nm (ε280) of W, Y and C. The
116 samples were analysed in quadruplicate.

117

118 *2.4. Human gastrointestinal juices*

119

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

128

129 *2.5. Ex vivo gastrointestinal digestion of β-casein variants*

130

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

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142

143 *2.6. Protein hydrolysis profile by sodium dodecyl sulphate polyacrylamide gel*
144 *electrophoresis*

145

146 The undigested and digested β-CN was mixed (1:1) with fresh sodium dodecyl
147 sulphate (SDS) buffer containing 5 mM dithiothreitol (DTT) and heated at 95 °C for 5 min.
148 Ten microlitres of sample was loaded in each well of a 10% Mini-PROTEAN TGX Stain-
149 Free Precast Gel (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK), and run at
150 200 V for 25 min. Low molecular mass protein ladder was used as a standard. The proteins
151 were fixed in 20% methanol and stained in Comassie Brilliant Blue (Bio-Rad Laboratories
152 Ltd), then de-stained and kept in preservation solution (10% glycerol and 10 % methanol).
153 Images were captured by Gel Doc EZ Imager (Bio-Rad Laboratories Ltd).

154

155 *2.7. Peptide identification of digested β-casein by liquid chromatography-electrospray*
156 *ionisation-tandem mass spectrometry ion trap*

157

158 The peptide analyses were performed with one of the two independent experiments
159 from GI digestion. Samples were diluted in ammonium bicarbonate (0.05 M) to an initial CN
160 concentration of 1 mg mL^{-1}, before being reduced (by DTE, at 60 °C for 10 min) and
161 alkylated (by iodoacetamide, at 37 °C for 30 min in dark). Formic acid (6.25 %) was added to
162 acidify the samples. The solution was filtered through a 10 kDa molecular mass cut-off spin-
163 filter (Millipore, Cork, Ireland) by centrifugation at 14,000 × *g* and at 4 °C for 10 min. All
164 samples were diluted 3-fold in 0.1% formic acid prior to LC-ESI-tandem MS analysis.
165 The digests were analysed on an Aeris Peptide C18 column of dimensions 250 mm ×
166 2.1 mm, with a particle size of 3.6 µm (Phenomenex, Torrance, CA, USA) of an Agilent LC

167 1200 series operated at 40 °C, and directly connected to an HCT Ultra Ion Trap (Bruker
168 Daltonics, DE, USA). A linear LC gradient, consisting of solvent A (0.1 % formic acid) and
169 B (90% acetronitrile, 0.1% formic acid), was set as: 0–40% B over 80 min and increasing to
170 80% B over 15 min, with flow rate at 200 µL min^{-1}. Sample injection volume was 5 µL. The
171 mass scan for MS mode was from 250 to 1800 *m/z* and MS/MS mode was from 100 to 1800
172 *m/z*. DataAnalysis (version 4.0) and Biotools (version 3.1) (Bruker Daltonic) were used to
173 process the MS/MS spectra. The data was then sent to Mascot (Matrix Science, MA, USA)
174 and searched against a custom in-house database with search parameters as follows: *Bos*
175 *Taurus,* none for proteolytic enzyme, and no modifications for identification of peptides.
176 Peptide hits above the Mascot score significant threshold ($P < 0.05$) were accepted, and
177 peptides of interest below threshold were manually analysed.
178

179 *2.8. Quantification of β-casomorphin-7 by multiple reaction monitoring mass*
180 *spectrometry*
181

182 The digests were analysed on a 1260 Infinity LC system (Agilent Technologies,
183 Waldbronn, DE, USA) coupled to a 6460 Triple Quad (QQQ) mass spectrometer (Agilent
184 Technologies). BCM7 was separated on a C18 column (2.1 mm × 50 mm, 1.8 µm, Agilent
185 Technologies) at 45 °C. The mobile phases contained (A) 0.1% formic acid in Milli-Q water
186 and (B) 90% acetonitrile in Milli-Q water with 0.1% formic acid at a flow rate of
187 550 µL min^{-1}. The gradient was as follows: 0–40% B over 18 min and increasing to 80% B
188 over 5 min. The injection volume was 10 µL. The 6460 QQQ was operated in multiple
189 reaction monitoring (MRM) mode with dwell time of 200 ms and fragmentor of 165 V. A list
190 of Q1/Q3 masses of BCM7 (790.2/383.1 and 790.2/530.0) was submitted as a batch for data
191 acquisition. The optimal collision energy (CE) was 30 eV. Serial standard concentration was

192 25, 50, 75, 125 and 250 fmol μL^{-1}. Isotope labelled (C^{13} and N^{15} at F) internal standard with
193 the concentration of 50 fmol μL^{-1} was spiked into the standard solutions and the digested
194 samples. Standard peptide BCM7 and isotope labelled BCM7 was purchased from
195 ThermoFisher Scientific (Biopolymers, Ulm, Germany) as AQUA Ultimate. Purity of these
196 peptides was above 97%. Quantification was calculated based on ratio of the analyte and
197 internal standard by MassHunter Quantitative Analysis software (Agilent Technologies). The
198 MRM method was validated for linearity, repeatability, limit of detection (LOD) and limit of
199 quantification (LOQ). The samples were measured in quadruplicates.
200
201 **3. Results and discussion**
202
203 *3.1. Purification of the β-casein variants*
204
205 β-Casein from homozygous milk was isolated for the genetic variants A1, A2 and I.
206 Chromatograms from LC-ESI/MS provided the molecular mass of the proteins in the purified
207 samples, which enabled the identification of the β-CN variants by confirming the
208 homozygosity and genotype with masses of 24,018 Da, 23,978 Da and 23,960 Da for variants
209 A1, A2 and I, respectively (Fig. 1). Considering the relative amount of protein in milk, the
210 yield from purification of β-CN was calculated by the amount (mg) retrieved from the milk
211 samples, and from the electropherograms obtained from capillary electrophoresis of each
212 milk sample (data not shown). The yield was estimated to be between 16% (A1) and 30% (I),
213 which is expected due to the process of cold storage, where only the β-CN loosely bound to
214 the other CNs through hydrophobic interactions and released into the whey fraction by the
215 cooling procedure was obtained.
216 The purity of β-CN relative to total protein in the isolates was determined by LC-ESI-

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217 MS analysis and predicted absorbance at 280 nm, and varied from 90% to 93% (Table 1).
218 These data are comparable with previously reported results by Petrat-Melin et al. (2015), who
219 reported purity of ≈ 90% with the same method for purification. This method is useful as it
220 combines both predicted and measured absorbance, which gives a better estimation of the
221 absolute protein content. Purification of β-CN by cold storage and ultracentrifugation is
222 simple and limits the risk of changing the physiochemical properties of the proteins, as could
223 occur with urea-based methods (O'Mahony & Fox, 2013; Petrat-Melin, 2014).
224

225 *3.2. Digestion of β-CN*

226

227 Purified β-CN was digested according to the INFOGEST protocol (Minekus et al.,
228 2014), with some modifications due to the use of human enzymes. The human digestive
229 juices comprise various enzyme isoforms, and this may broaden their spectrum of cleavage
230 (Ulleberg et al., 2011). Subsequently, the enzyme activities in the human juices were
231 calculated on the basis of the ratio between substrate and enzyme secretion (v/v) in an "in
232 house" protocol. Eriksen et al. (2010) conducted a study on digestion with porcine and
233 human GI enzymes and concluded that the human juices perhaps should be preferred over
234 pure commercial enzymes when simulating human digestion, as they observed differences in
235 the degradation pattern of the proteins by human juices compared with commercial enzymes.
236 A preliminary study of the digested proteins by sodium docecylsulphate-
237 polyacrylamide gel-electrophoresis (SDS-PAGE) was done to ensure that the enzyme activity
238 was sufficient for hydrolysis of proteins and compared with earlier data from Islam, Ekeberg,
239 Rukke, and Vegarud (2015). The degradation profiles illustrated in Fig. 2 proved that the
240 time chosen in the gastric and duodenal phases were sufficient for enzymatic digestion of
241 β-CN. Large variation in gastric transit times of 15 min to 3 h have been recorded, depending

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242 on the texture and viscosity of the food bolus (Guerra et al., 2012), and the intestinal phase
243 can last up to 5 h. Furthermore, the INFOGEST protocol states that 2 h in each phase is
244 sufficient for digestion, as it represents half-emptying time of a moderately nutritious and
245 semi-solid meal (Minekus et al., 2014). However, in the study performed by Islam et al.
246 (2015), CNs showed almost complete degradation after 40 min in the gastric phase with
247 human gastric juice, and the remaining proteins (whey) were degraded after 5 min in the
248 duodenal phase with human duodenal juice. These results laid the basis for the chosen
249 digestion time in our study.

250 As illustrated in Fig. 2, SDS-PAGE did not reveal any obvious, visual differences in
251 the degradation pattern among the variants. After initial 60 min of gastric digestion, the major
252 fraction of β-CN was digested and only small faint bands were visible around < 20 kDa. The
253 visible bands around 30–60 kDa (a and b) represented the human duodenal enzymes, reported
254 by Devle et al. (2014). It is expected that the genetic variants can produce different peptides,
255 however, these peptides may be of low concentration and smaller than 10 kDa, and is
256 therefore not visible in the gel. Some faint bands around 10–20 kDa in the gel were visible
257 after 60 min of gastric digestion. These bands may represent degraded β-CN, or it could
258 represent traces of whey proteins that were detected in the isolated β-CN after purification.
259

260 *3.3. Peptide identification by mass spectrometry*

261

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

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

280 Furthermore, during the gastric phase of digestion, the active enzyme pepsin
281 hydrolyses the proteins. This enzyme cleaves preferably at sites of F, L and Y (Rawlings,
282 Barrett, & Finn, 2016); however, pepsin can cleave with a more complex action as well,
283 depending on the AA combination upstream or downstream of the cleavage site (Tang,
284 1963). Several sites of cleavage were observed after digestion, including at residue P and the
285 average length of all identified peptides decreased from thirteen AA in the gastric digestion
286 to an average length of nine AA at the end of duodenal digestion. P is considered rather
287 resistant to proteolytic cleavage, thus the complex mixture of different isoforms of GI human
288 juices may have enhanced the diverse cleavage.

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

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

303 Gastrointestinal digestion of β-CN resulted in identification of peptides previously
304 described as bioactive, and is presented in Table 4. In the gastric phase of digestion two
305 BAPs were identified: the ACE-inhibitory peptide ^{193}YQEPVLGPVR^{202} was identified from
306 variants A1 and A2, and the antimicrobial and immunomodulatory peptide
307 ^{193}YQEPVLGPVRGPFPIIV^{209} was identified from all three variants. In the duodenal phase
308 of digestion the ACE-inhibitory peptide ^{6}LNVPGEIVE^{14}, previously reported by Gobbetti,
309 Stepaniak, De Angelis, Corsetti, and Di Cagno (2002), was also identified from all variants.
310 However, one peptide exclusively identified from the A2 variant after 120 min duodenal
311 digestion was ^{60}YPFPGPIPN^{68}. This is a BAP reported by Saito, Nakamura, Kitazawa,
312 Kawai, and Itoh (2000), where the residues I^{66} and P^{67} at the C-terminus of the peptide
313 showed potent ACE-inhibitory effect. However, the author reported that this peptide had
314 rather low anti-hypertensivity, which is explained by the large size of the molecule, that
315 requires further digestion by intestinal enzymes before absorption. Moreover, the ACE-
316 inhibitory and anti-oxidative peptide ^{59}VYPFPGPIPN^{68} was observed after duodenal

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

323 The ACE-inhibitory peptide ^{133}LHLPLP^{138}, previously reported by Quirós et al.
324 (2007), was identified from both variant A1 and A2. This peptide has shown to be resistant to
325 gastrointestinal digestion, but hydrolysed to its active form ^{134}HLPLP^{138} by brush border
326 peptidases in the intestinal epithelium prior to absorption (Quirós, Dávalos, Lasunción,
327 Ramos, & Recio, 2008). However, in our study this pentapeptide was identified already after
328 5 min digestion in the duodenal phase, which also indicates the broad activity of the human
329 digestive juices. Moreover, two derived peptide fragments from hydrolysis of the
330 aforementioned hexapeptide have also previously been detected in plasma; i.e., the
331 tetrapeptide fragments ^{134}HLPL^{137} and ^{135}LPLP^{138} were detected 5 min after oral
332 administration of HLPLP in rats (Sánchez-Rivera et al., 2014). In addition, the tripeptide
333 ^{134}HLP^{136} was detected with a rising concentration of the peptide until 60 min of incubation
334 in plasma (Sánchez-Rivera et al., 2016).

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

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

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

362

363 *3.4. Quantification of BCM7*

364

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

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

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

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

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

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

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

413 Despite the fact that BCM7 was detected after digestion of β-CN with human GI
414 enzymes, it may not be absorbed intact in the intestines, and if it is absorbed, it may still be
415 hydrolysed by brush border peptidases (e.g., dipeptidyl peptidase-4) before reaching its target
416 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

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442 therefore be more prone to physiological effects of BAPs due to a higher permeability of the
443 intestinal wall. De Magistris et al. (2010) found that the intestinal permeability was abnormal
444 in children suffering from autistic spectrum disorders. However, in other studies evaluating
445 food-derived opioids in urine output of children, there was no evidence linking autism to
446 opioid peptides or to DPP4 deficiency (Cass et al., 2008; Hunter, O'Hare, Herron, Fisher, &
447 Jones, 2003).
448

449 **4. Conclusion**

450

451 The use of human enzymes in simulation of human digestion has led the research one
452 step forward towards in vivo digestion, with an ex vivo approach. The present study showed
453 that different genetic variants of β-CN can affect the hydrolysis by gastrointestinal proteases,
454 thus affecting peptides formed. It has also been established that ex vivo digestion of β-CNs
455 leads to generation of several BAPs; however, the release of BCM7 is potentially not solely
456 dependent on the genetic variants with residue H at position 67 in the AA sequence of β-CN.
457 We have identified BCM7 from all variants, and the difference in the concentration between
458 A1 and A2/I was rather low. These results imply that when studying release of BAPs during
459 digestion, human enzymes should be preferred. Moreover, there is a need for evaluating the
460 bioavailability of generated BAPs, as their function after release in the intestines is rather
461 ambiguous. Irrespective of the on-going debate regarding health effects of BCM7 and other
462 peptides, the data attained in this study brings additional clarification on the possible
463 generation of BCM7, but the results still need to be confirmed by in vivo studies.
464

465 **Acknowledgement**

466

467 The technical assistance from Gitte Hald Kristiansen is gratefully acknowledged.

468

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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

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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.

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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).

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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.

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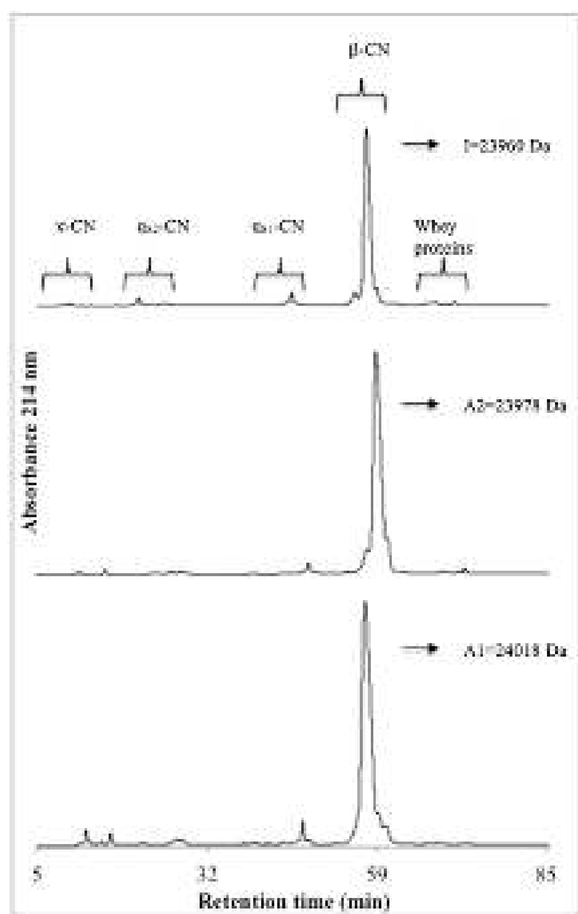

Figure 1

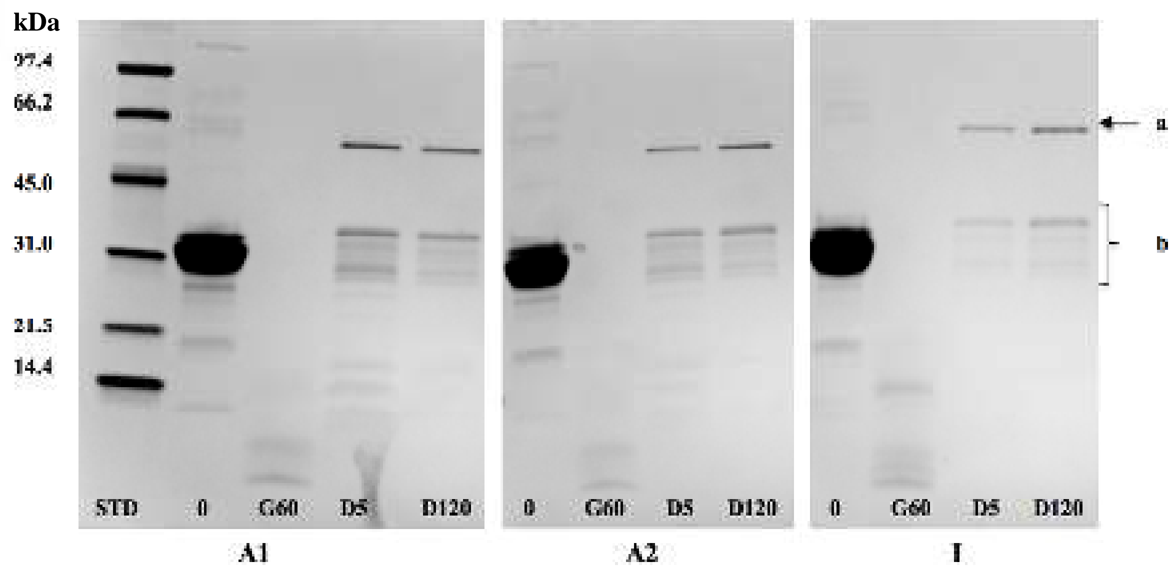

Figure 2.

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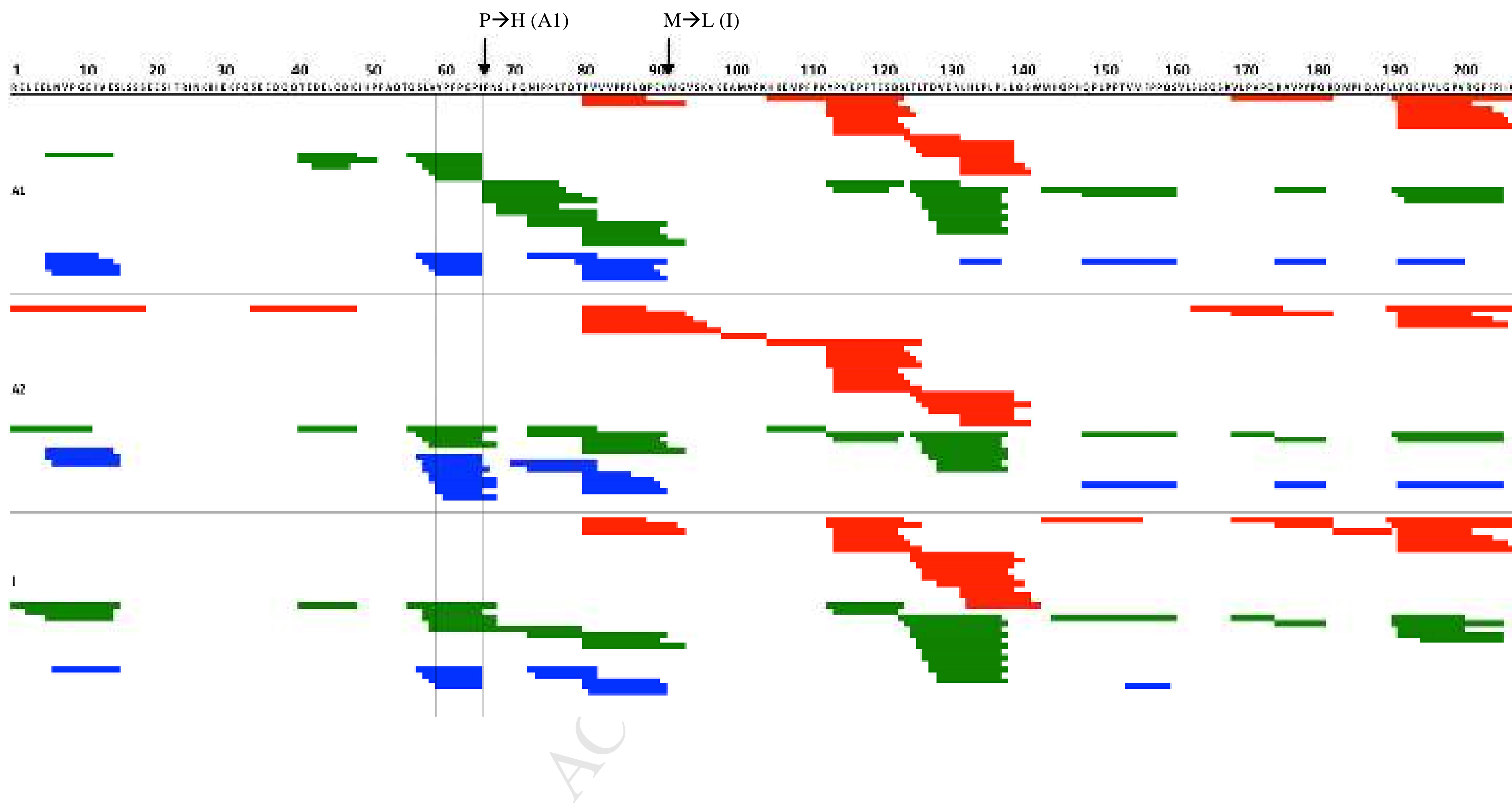

Figure 3

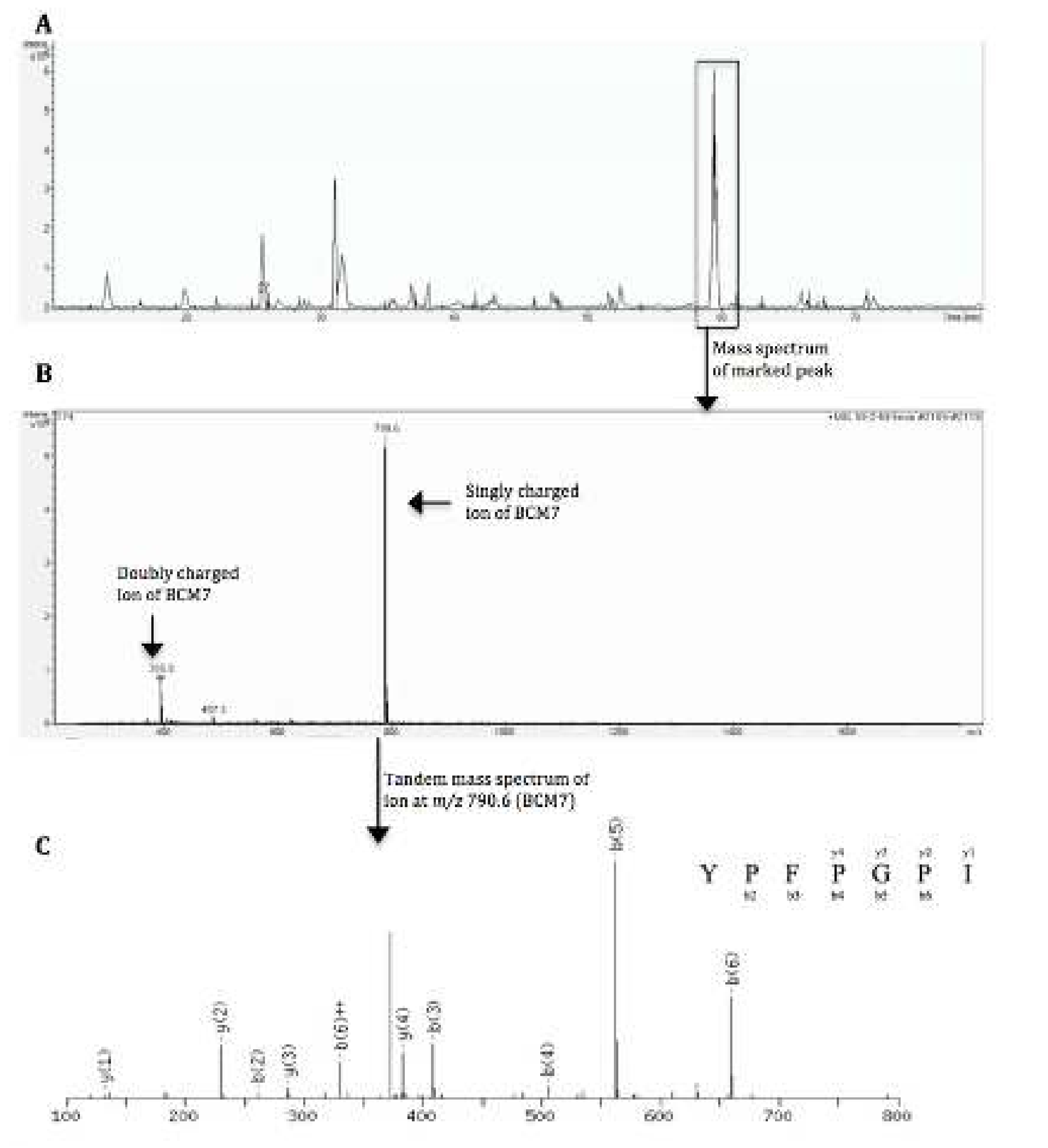

Figure 4.

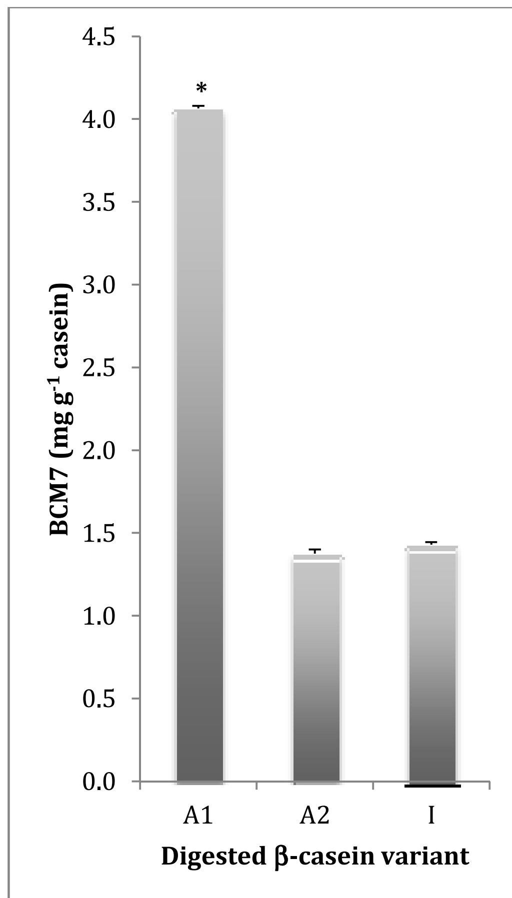

Figure 5.

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