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Ex Vivo Digestion of Bovine Milk with Genetic Variants A1 and A2 of β-Casein and Identification of Bioactive peptides

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Preface

This thesis is performed as part of a two-year master program in Food Science at the Norwegian University of Life Sciences (NMBU). The project was performed with the research group in Food Proteins – structure and biological function at The Department of Chemistry, Biotechnology and Food Science (IKBM). The majority of the laboratory work was performed at the Department of Food Science at Aarhus University in Denmark. The e*x vivo* digestion was performed at IKBM.

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Abstract

Milk-derived bioactive peptides (BAPs) have shown to possess physiological effects linked to the development of non-communicable diseases, such as cardiovascular disease, cancer and diabetes. These peptides lie within the sequence of the precursor protein, and can be released by enzymatic proteolysis during gastrointestinal digestion. As proteolytic enzymes have preferences for cleavage at specific amino acids, genetic polymorphism can affect the release of BAPs during digestion, due to amino acid substitution. Bioactive properties within a protein are therefore suggested to be dependent on the genetic variants.

The aim of the study was to investigate whether different variants of β-casein (CN), such as A1 and A2, give rise to peptides of different lengths and with different bioactivities during digestion. β-Casein was purified from bovine milk with homozygous genetic variant A1, A2 and I, and the heterozygous type A2F. Skimmed milk and purified β-CN were digested in a static *ex vivo* model with human gastrointestinal juices, with subsequent identification of peptides released. Sampling was done after 30 and 60 min of gastric digestion, and after 5, 30 and 120 min of duodenal digestion. Degree of hydrolysis (DH) was measured at for each sampling point, and showed to increase substantially in the duodenal phase; however, there were no significant differences ($p<0.05$) in the DH of the genetic variants of β-CN. Fractions of undigested proteins remaining in the gastric and duodenal phase were analysed by capillary- and gel electrophoresis. The digestion pattern showed high degradation of CNs and high resistance of whey protein degradation after 60 min of gastric digestion. All proteins were degraded after duodenal digestion. Mass spectrometry analyses of peptides released during digestion revealed that amino acid substitution affected the proteolytic hydrolysis to some extent, where the peptide f(60-68) was exclusively identified from variants containing proline at position 67. Most strikingly, the opioid peptide BCM7, f(60-66), was identified from both A1 and A2 genetic variant of β-CN after 120 min duodenal digestion. In conclusion, genetic variation of β-CN may affect the proteolysis during digestion; however, the release of BCM7 does not seem to be linked solely to variant A1 as previously suggested in the literature. Further quantification of BCM7 formed during digestion is needed to establish any significant differences between the variants.

Sammendrag

Bioaktive peptider (BAP) som stammer fra melk har vist seg å besitte fysiologiske egenskaper forbundet med utvikling av ikke-smittsomme sykdommer som hjerte- og karsykdommer, kreft og diabetes. Disse peptidene ligger forplantet i det intakte proteinet, og kan bli frigjort fra den native sekvensen ved proteolyse under fordøyelse. Ettersom proteolytiske enzymer besitter spesifikke preferanser for kløyving kan proteiners polymorfisme påvirke frigjørelse av bioaktive peptider grunnet aminosyresubstitusjon i proteinsekvensen. Proteinets bioaktive egenskaper er derfor antatt å være avhengig av den genetiske varianten av proteinet.

Hensikten med denne studien var å undersøke om ulike varianter av β-kasein (KN), A1 og A2, påvirker frigjørelse av peptider etter fordøyelse. β-Kasein ble renset fra kumelk homozygot for variant A1, A2 og I, i tillegg til heterozygot A2F. Renset β-KN og skummet melk ble fordøyd i en statisk *ex vivo* modell med human mage- og tarmsaft, med påfølgende identifisering av frigitte peptider. Uttak ved forskjellige fordøyelsestrinn ble gjort etter 30 og 60 min i magefasen, og etter 5, 30 og 120 min i tarmfasen. Hydrolysegraden ble undersøkt for vært fordøyelsestrinn og viste en markant økning fra mage- til tarmfasen. Det var derimot ingen signifikant forskjell ($p<0.05$) i hydrolysegrad mellom de ulike genetiske variantene av β-KN. Ufordøyde proteiner ble undersøkt ved hjelp av kapillær- og gelelektroforese. Nedbrytningsgraden av KN var høy, mens myseproteinene var relativt resistente mot nedbryting etter 60 min fordøyelse i mage. Alle proteiner var brutt ned etter fordøyelse i tarm. Massespektrometriske analyser av peptider frigitt under fordøyelse bekreftet at aminosyresubstitusjon påvirker proteolytisk hydrolyse i noen grad, der peptidet f(60-68) ble utelukkende identifisert fra β-KN variant med prolin i posisjon 67. Det mest oppsiktsvekkende var identifisering av peptidet BCM7, f(60-66), fra både A1 og A2 variant av β-KN etter 120 min fordøyelse i tarm. Resultatene tyder på at genetisk variasjon av β-KN kan påvirke proteolysen under fordøyelsen, derimot viser frigjøring av BCM7 ikke å være avhengig av β-KN variant A1. Videre kvantifisering av identifisert BCM7 etter fordøyelse er nødvendig for å påvise om det er en signifikant forskjell mellom de genetiske variantene.

Abbreviations

ACE – Angiotensin-1 converting enzyme

BAP – Bioactive peptide

BBB – Blood-brain barrier

BCM – Beta-casomorphin

CE – Capillary electrophoresis

CN – Casein

CNS – Central nervous system

DH – Degree of hydrolysis

DPP4 – Dipeptidyl peptidase-4

EFSA – European Food Safety Authority

ESI – Electrospray ionization

GI – Gastrointestinal

GIT – Gastrointestinal tract

HDJ – Human duodenal juices

HGJ – Human gastric juices

RP-HPLC – Reversed phase High performance liquid chromatography

LAB – Lactic acid bacteria

LDL – Low-density lipoprotein

MS – Mass spectrometer

SDS – Sodium-Dodecyl Sulphate

T1D – Type 1 diabetes mellitus

TAME – p-toluene-sulfonyl-L-arginine methyl ester

TCA – Trichloro acetic acid

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1 Introduction

1.1 General Background and Theory

The human body is dependent on proteins to function. The protein's role is vital for biochemical reactions, the immune system, metabolism and structure and strength of the cells. Proteins **are** an important part of the human diet, through various animal and plant-derived sources. Peptide sequences encrypted within the intact protein may exert physiological functions in the human body after enzymatic release during digestion (Foltz et al. 2008). Furthermore, these peptides can interact with receptors on target cells and induce biological activities including antioxidant, antihypertensive, opioid, antimicrobial and immunomodulatory. Consequently, bioactive peptides (BAPs) have been linked to various non-communicable diseases such as cancer, diabetes, autoimmunity, and cardiovascular disease (Osborne et al. 2014). Understanding the complex activity of bioactive peptides, and how this affects the human health, has been approached for several decades, and the on-going research is still showing diverse results. In 2009 the European Food Safety Authority (EFSA) published a review on the potential health effects of β-casomorphins-7 (BCM7) and related peptides. This peptide is encrypted in the native sequence of β-casein (β-CN) and can be released through enzymatic hydrolysis after milk ingestion. Furthermore, the possible health effects associated with BCM7 have been applied in the milk industries. *The a2 Milk Company* was founded in New Zealand in 2000 and has expanded to Australia, US, UK and China since then. The company distributes pure A2 milk, which is milk from cows homozygous for the A2 genetic variant of β-CN. This milk is therefore free from the A1 genetic variant that is suggested to lead to the formation of BCM7 during digestion. A number of *in vitro* and *in vivo* studies have described the effect after A1 β-CN ingestion (De Noni 2008; Jinsmaa & Yoshikawa 1999). Atherosclerosis development has been detected in a rabbit model after digestion of A1 β-CN (Tailford et al. 2003), and the development of insulin-dependent diabetes have been detected in mice fed A1 β-CN. After thorough investigation of new scientific literature, EFSA concluded that a formal risk assessment of BCM and other food-derived peptides was not recommended (EFSA 2009).

1.2 Milk as a Nutrient Source

Milk is an essential source for nutrients, containing a proportional balance between the macronutrients protein, lipid and carbohydrate, together with a wide range of minerals, vitamins and bioactive peptides. As milk contains all the essential amino acids, it is a great source of nutrients, especially for early infant development, where the only source of nutrition is from the mother milk or milk-based formulas. The nutrient content of milk from different species reflects the requirements of the mammals' offspring. For instance, the human milk has a different ratio of casein (CN) and whey (40:60, respectively), compared to bovine milk (80:20, respectively), as CNs are more difficult to digest for human infants than whey (Becker et al. 1976). Furthermore, bovine milk contains a higher amount of total protein than human milk since calves need a rapid growth, whereas the human milk has a higher content of fat, for the early development of brain and spinal cord of the infant. Today, milk proteins are considered an important source of bioactive peptides, but such peptides are also found in egg, meat and plants (Korhonen & Pihlanto 2003). These peptides can carry out a spectrum of different reactions in the human body. Some of these will be discussed more in detail in chapter 1.5.2.

The diverse content of nutrients in milk has led to the recommendation of milk consumption on a daily basis. The Norwegian directory of health (Helsedirektoratet) provides information on recommended food intake (Helsedirektoratet 2014). They do not provide specific amount of milk intake, however, they recommend that low-fat dairy products should be a part of the daily diet. The dietary guidelines for Americans produced by the U.S. Department of Agriculture and the U.S. Department of Health and Human Services (2010) recommend a daily intake of three cups of low-fat milk for adults.

1.3 Milk Proteins

The most abundant proteins in bovine milk are the caseins. They constitute approx. 80 % of the total protein content, where β-CN together with α_{S1}-CN make up 60 % of the total protein content. The whey proteins α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) together with some minor proteins constitute the remaining 20 % of proteins in milk. The relative amount of the different bovine proteins is listed in Table 1.

Table 1. The relative amount of proteins in bovine milk. Adapted from Walstra et al. (2005)

Protein	% of total protein in milk
α_{S1}-casein	32
α_{S2}-casein	8.4
β-casein	28.4
κ-casein	9.3
α-lactalbumin	3.7
β-lactoglobulin	9.8
Other/minor proteins	8.4

1.3.1 Structure and Physiochemical Properties of Casein

For the separation and isolation of CNs, it is important to understand the structure and the physiochemical properties of the CN micelle, and how the different CNs are bound together. α_{S1}- and α_{S2}-CN interact through phosphorylation on their serine groups and form nanoclusters together with β-CN, away from the micelle surface. The β-CN is also loosely bound to other CNs through hydrophobic interactions. The κ-CN, however, is located on the micelle surface with its glycosylated tail (Dalgleish & Corredig 2012) (see Figure 1). When CN is held at 4 °C the hydrophobic interactions between the β-CN and the CN micelle are weakened and the β-CN dissociates from the micelle. Ultracentrifugation (>100 000 *g*) of milk samples separates the soluble phase, containing the dissociated β-CN and whey proteins, from the colloidal phase, containing the remaining CNs bound in micelles (Petrat-Melin et al. 2015). Further separation of β-CN is achieved by acidification to pH around 4.6, the isoelectric point of CN. At this pH the molecule is neutral and there is no repulsive forces between the molecules, and the protein will precipitate. Centrifugation of the acidified CN in the soluble phase makes it possible to isolate the β-CN from whey proteins.

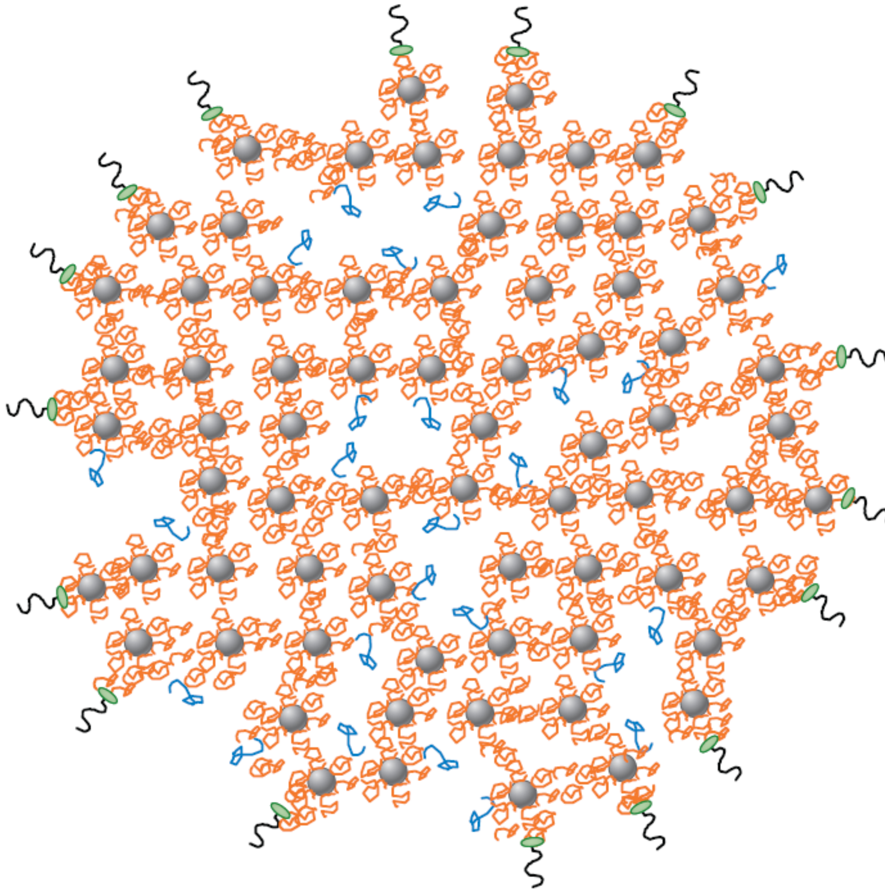

Figure 1. Illustration of the casein micelle: αS1-, αS2- and β-CN are illustrated in orange, binding calcium (grey) with their phosphate groups, forming nanoclusters. The remaining β-CN (blue) is located inside the micelle structure, away from the water surface, forming hydrophobic interactions. The κ-CN (green) is located on the surface of the micelle with its glycosylated tail. Adapted from Dalgleish and Corredig (2012).

The traditional separation of CNs has been done by addition of urea to unfold the micelle structure, with subsequent acid precipitation (Hipp et al. 1952). However, the use of urea may result in interaction of CN with cyanate, which could cause modification of the side-chains and alter the action of digestive enzymes (Fox & McSweeney 1998; Petrat-Melin 2014). Other methods for CN precipitation can be achieved with salting-out methods or addition of calcium chloride. These methods are mainly used for separation of CNs from whey proteins, however calcium precipitation can be used to separate κ-CN from the other CNs, due to its calcium insensitive properties (Dalgleish & Corredig 2012).

1.3.2 β-casein Polymorphism

There are four casein genes that are tightly linked in a 250-kb cluster. β-Casein is encoded by the CSN2 gene on chromosome 6. This gene is highly polymorphic and gives rise to various genetic variants of β-CN (Caroli et al. 2009). Twelve different genetic variants of β-CN have been identified in cattle breeds (A1, A2, A3, B – J), where variant A1 and A2 is of highest frequency. The difference between these variants is the amino acid in position 67 (sequence shown in Figure 2). Variant A2 contains Proline (P, the amino acids will be referred to with one letter abbreviations, see appendix) at this position, whereas in variant A1 it is substituted by H. This is due to a single nucleotide polymorphism by substitution at codon 67 in the nucleotide sequence of β-CN, where CCT results in P (variant A2) and CAT results in H (variant A1) (Kamiñski et al. 2007). This amino acid substitution alters the ability of enzymatic cleavage due to conformational differences in the secondary structure of the protein. The P residue in A2 is suggested to prevent cleavage of the peptide bond at this position, whereas the H in A1 allows cleavage of the perceiving seven amino acid residues to yield the peptide β-casomorphin-7 (BCM7), illustrated in Figure 3 (Clarke & Trivedi 2014). The amino acid substitutions for all variants of bovine β-CN are illustrated in Table 2.

```
        10         20         30         40         50         60
RELEELNVPG EIVESLSSSE ESITRINKKI EKFQSEEQQQ TEDELQDKIH PFAQTQSLVY

        70         80         90        100        110        120
PFPGPIPNSL PQNIPPLTQT PVVVPPFLQP EVMGVSKVKE AMAPKHKEMP FPKYPVEPFT

       130        140        150        160        170        180
ESQSLTLTDV ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY

       190        200
PQRDMPIQAF LLYQEPVLGP VRGPFPIIV
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Figure 2. Amino acid sequence of bovine A2 β-CN.

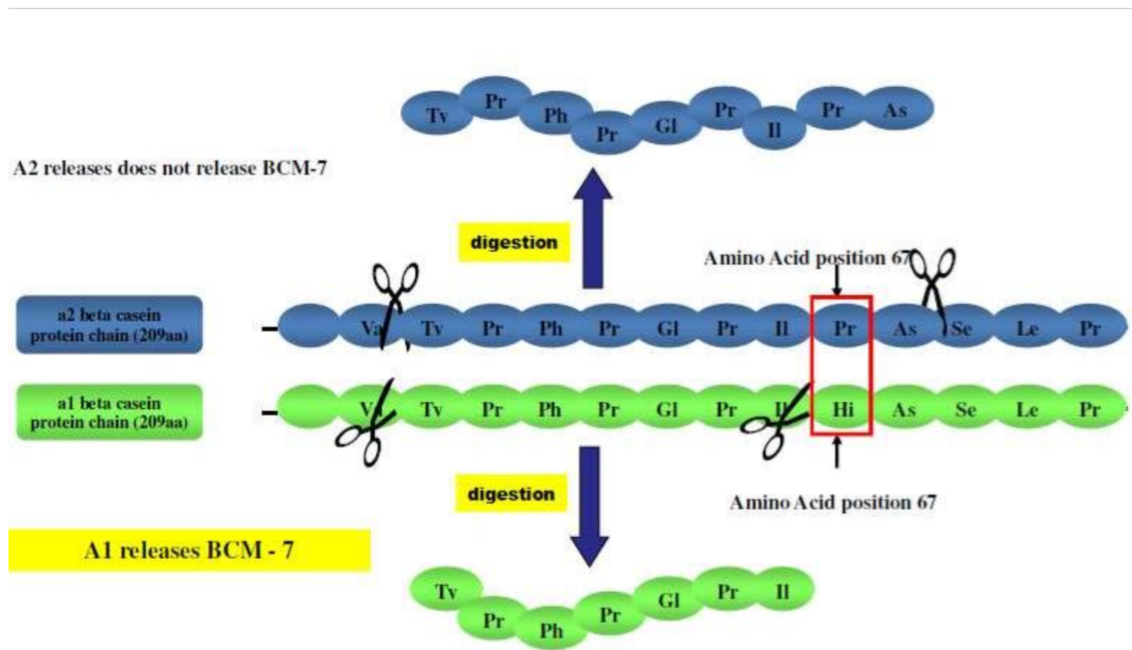

Figure 3. Cleavage of β-casein by proteolytic enzymes and release of β-casomorphin-7. Adapted from Clarke and Trivedi (2014).

Table 2. Amino acid substitutions in the β-casein protein, adapted from Caroli et al. (2009), and McSweeney and Fox (2013)

	Change in amino acid sequence (position)											
β-casein genetic variant	18	35	36	37	52	67	72	93	106	122	138	152
A2	S-P	S-P	E	E	F	P	Q	M	H	S	P	P
A1						H						
A3									Q			
B						H				R		
C		S		K		H						
D	L											
E			K									
F						H						L
G						H					L	
H							E	L				
I								L				
J					S							

1.4 Digestion of Proteins

The gastrointestinal tract (GIT) is comprised of several organs and extends from the mouth to the anus, illustrated in Figure 4. The GIT can be divided into two tracts. The upper tract consists of the oral cavity, oesophagus, stomach, duodenum, jejunum and the ileum. The lower tract consists of the cecum, colon, rectum and the anal canal. The mechanical and physiological machinery in the GIT is dependent on the food consistency and the nutrient content ingested. The food is masticated in the oral cavity where the secretion of saliva starts (Verhoeckx et al. 2015). The saliva contains amylase, which will start the digestion of amylose- and amylopectin-containing nutrients, such as starch. The mastication of food forms a bolus that is transported through the oesophagus to the stomach. The pH in the stomach is gradually lowered to pH 1-2 due to acid secretion, which gives the optimal pH for the gastric enzyme to digest the food (Saladin 2012). The low pH of the gastric acid enables the conversion of pepsinogen into active pepsin, it reduces the survival of some foodborne bacteria, and it results in denaturation of proteins.

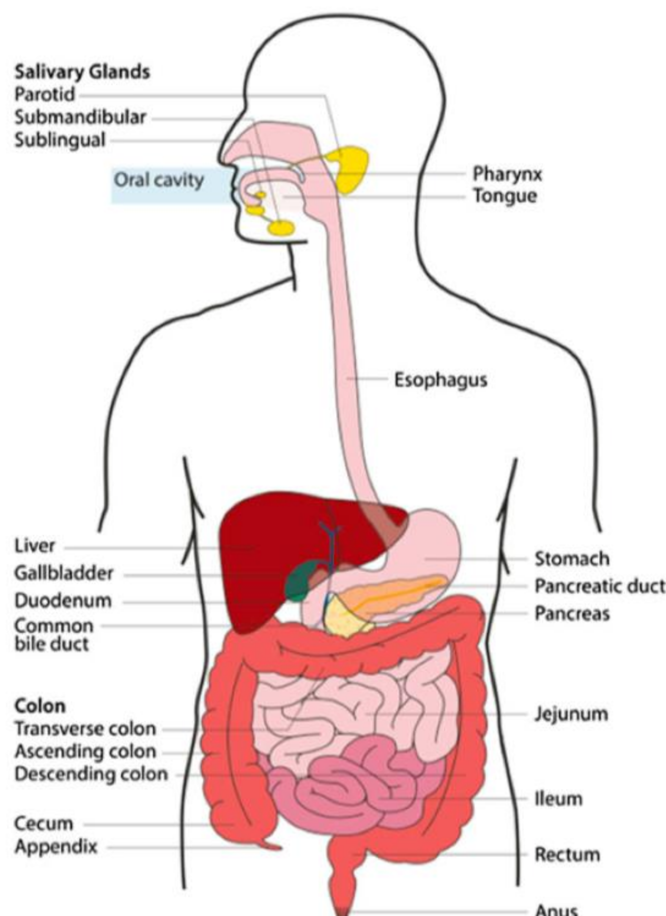

Figure 4. The digestive system (Verhoeckx et al. 2015).

The digestion of protein starts in the stomach, where pepsin breaks down the protein into smaller peptides by breaking the peptide bonds in the amino acid chain. Pepsin will hydrolyse internal peptide bonds of ingested proteins, preferably at sites of aromatic and hydrophobic amino acids, such as Y, F and L (MEROPS 2016). Pepsin exhibits maximal activity at pH 2.0 and is inactivated at pH 6.5, which is attained in the duodenum. When the semi-digested peptide mix reaches the duodenum, the intestinal enzymes continue to break down the peptides. Trypsinogen is secreted from the pancreas and is converted via an enteropeptidase to trypsin by proteolytic cleavage, when it reaches the duodenum. Trypsin cleaves C-terminally to K and R, chymotrypsin cleaves C-terminally to aromatic and bulky amino acids, and elastase cleaves C-terminally to A, V and to some extent L (MEROPS 2016). The pH in the duodenum is gradually increased to 5-7.5, due to the secretion of bicarbonate and pancreatic juices. This inactivates the gastric enzymes, and gives the optimal activity for the duodenal enzymes. The final stage of digestion of proteins occurs on the surface of intestinal enterocytes, by brush boarder enzymes, where peptides are hydrolysed to amino acids, di- and tripeptides (Verhoeckx et al. 2015). The nutrients are then absorbed by the enterocytes of the jejunum and ileum and can be further degraded by intracellular proteases before entering the blood stream. Absorption of water and fermentation of complex nutrients occurs in the large intestine. These nutrients are mostly complex polysaccharides, which the human body cannot digest by its own digestive enzymes, but with the microbiota in the large intestine.

1.5 Bioactive Peptides

Bioavailability of peptides is dependent upon three steps: 1) digestibility and solubility of the component in the GIT; 2) absorption of the component by the intestinal cells and transport into the circulation; and 3) incorporation from the circulation to the functional target (Verhoeckx et al. 2015). A bioactive peptide must therefore be activated to carry out bioactive functions. Within the parent protein peptides are inactive and need to be released from the native sequence to gain their function. This action occurs during digestion through hydrolysis by digestive enzymes or proteolytic activity derived from microorganisms, such as lactic acid bacteria (LAB), which exist naturally in the GIT or in the ingested food. Milk is a precursor of many bioactive peptides (BAPs) (Nongonierma & FitzGerald 2015). Research shows that milk-derived BAPs have antihypertensive and antioxidative properties, and they can carry out a number of reactions if they reach their final target after absorption and transportation (Clausen et al. 2009; Fitzgerald & Meisel 2000; Korhonen & Pihlanto 2006).

Even before absorption, BAPs can influence the environment in the GIT. BAPs can also target an immune response or bind to opioid receptors. Figure 5 illustrates the diverse functions of milk-derived BAPs. The milk-derived peptides will be further discussed in the following chapters, with emphasis on the β-casein-released β-casomorphin-7.

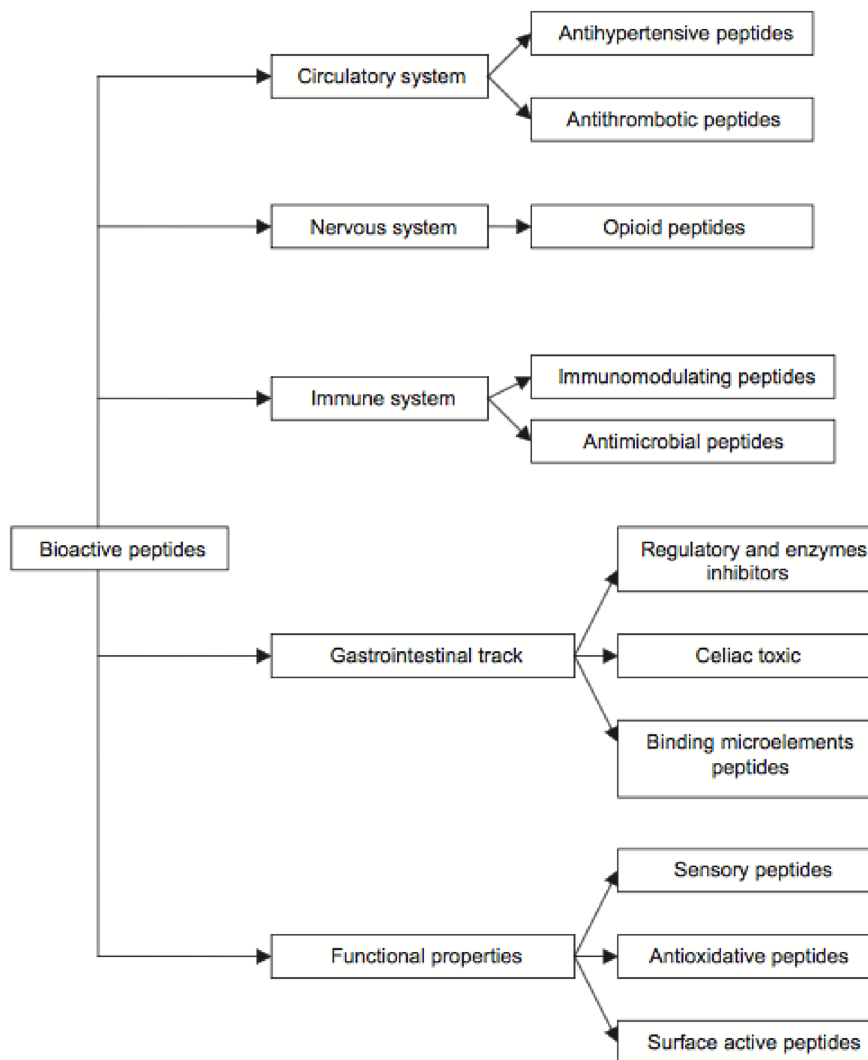

Figure 5 Function of bioactive peptides originated from bovine milk. Adapted from Dziuba and Dziuba (2014).

1.5.1 Absorption and Transport of BCM7 and Other Milk-Derived Peptides

Beta-casomorphin-7 (BCM7) can influence a number of activities in the human body. The highly ubiquitous expression of opioid receptors in the body promotes the ability of BCM7 activation. However, the activation of BCM7 after food ingestion is dependent on the

peptides ability to cross the gastrointestinal (GI) wall (EFSA 2009). Passive diffusion is the most likely mechanism for this to occur (Foltz et al. 2008); nevertheless, the extent of this could be small, as passive diffusion rather transports single amino acids or di- and tripeptides. Beta-casomorphin-7 is a heptapeptide and will not be easily transported under normal conditions. In individuals with "leaky gut" the intestinal permeability is significantly increased, due to damage of the intestinal wall, and the capacity of substances to cross the GI wall is therefore higher.

In addition to crossing the GI wall, the opioid peptides are suspected to cross the blood-brain barrier (BBB) to activate the central nervous system (CNS) (Ganapathy & Miyauchi 2005). Peptides can bind weakly to carrier proteins, which can protect them from hydrolysis. However, the blood contains peptidases that can affect the bioavailability of peptides in the blood. Furthermore, the endothelial cells forming the BBB are equipped with tight junction and proteolytic enzymes that can degrade peptides. If a peptide, such as BCM7 escapes the mechanisms for degradation, the peptide can bind as a substrate to the Peptide Transport System called PTS-1 in the BBB. As the peptide has been transported into the brain, it takes part in a large amount of reactions. Milk-derived bioactive peptides have shown diverse effects related to different non-communicable diseases, and the activities of which they can perform are enormous: As ACE-inhibitors and stimulators of low-density lipoprotein (LDL) oxidation, the peptides can be related to the development of cardiovascular diseases (Saito et al. 2000; Tailford et al. 2003). Beta-casomorphin-7 has also been related to the autistic spectrum disorders (ASDs), such as autism and Asperger's syndrome, through opioid receptors in individuals with leaky-gut (de Magistris et al. 2010). Furthermore, the peptides have shown to induce the production of autoantibodies that can cause auto-immediate killing of pancreatic beta cells, which in turn can cause the development of type 1 diabetes mellitus (T1D) (Elliott et al. 1997). However, according to the EFSA (2009) review, there is no significant proof that BCMs are correlated to these conditions.

1.5.2 Physiological Function of BCM7 and Other Milk-Derived Peptides

Beta-casomorphin-7 has shown to affect the GI transit time (GITT). A study performed on Wistar rats found that consumption of A1 β-CN increased GITT relative to A2 β-CN (Barnett et al. 2014). A prolonged transit in the GIT can cause increased fermentation of dietary components, which in turn can lead to abdominal discomfort such as bloating and

constipation. In addition to these results, the study found an increased activity of colonic myeloperoxidase after A1 β-CN administration. Myeloperoxidase produces hypochlorous acid that can mediate inflammation and cause oxidative damage (Daugherty et al. 1994). These findings are consistent with another study showing a significant increase in the expression of intestinal inflammatory molecules after A1 β-CN administration in mice (Ul Haq et al. 2014).

Beta-casomorphin-7 has also been linked to the pathogenesis of type 1 diabetes mellitus (T1D). A study performed by Elliott et al. (1999) showed strong correlation of diabetes incidence and the consumption of A1 β-CN. Data collected from Iceland significantly increased the hypothesis of the link between A1 β-CN consumption and diabetes. Iceland had the highest total milk protein consumption, despite the low incidence of childhood diabetes, which could be explained by the low A1 β-CN values in their native breeds (Elliott et al. 1999). Furthermore, A1 β-CN has shown to induce diabetes in non-obese diabetogenic mice via opioid receptors, while subjects administrated with naloxone (micro-receptor-specific antagonist) were prevented (Elliott et al. 1997). Despite these results, EFSA (2009) has reviewed the potential health impact on BCM7 and suggested that there was no significant proof of the links between T1D and BCM7 in humans. The conclusion was that more comprehensive studies are needed to eliminate other factors that can be responsible for this outcome.

The implication of food-derived peptides' effect on cardiovascular health has been reported in animal studies (Tailford et al. 2003). It has been suggested that BCM7 could be pro-atherogenic due to the peptides' ability to stimulate the oxidation of LDL. Tailford et al. (2003) found that the consumption of A1 β-CN increased the concentration of serum cholesterol, LDL and high-density lipoprotein (HDL) in a rabbit model. In addition, the authors found a higher percentage of the aorta surface area covered by fatty streaks in rabbits fed A1 β-CN, than those fed A2 β-CN. Low-density lipoprotein is an important lipid carrier in plasma and the increased concentration of lipids in the arteries can induce accumulation of white blood cells (fatty streaks). This will result in plaque formation of the arterial wall.

Several studies have shown that released milk-derived peptides may inhibit the angiotensin-converting enzyme (ACE) (Foltz et al. 2008; Miguel et al. 2009). The function of ACE

inhibitors is antihypertensive, which means it is a potent drug for treatment of high blood pressure (Petrillo Jr. & Ondetti 1982). Inhibitors of ACE block the conversion of Angiotensin I released from angiotensinogen by renin, into angiotensin II. There are several peptides shown to have this function, and the most evidenced ones are the tripeptides VPP and IPP, that corresponds to β-CN fraction 84-86 and 74-76, respectively (Hayes et al. 2007). In general, peptides having Y, F, W or P at their C-terminus showed higher inhibition against ACE.

As an opioid peptide, BCM7 can bind to receptors in the brain and directly modulate neurosignalling pathways, leading to the development of autism (Sokolov et al. 2014). The Y at the N-terminal and the aromatic F in the third position gives BCM7 an important structural motif for binding to opioid receptors (Nagpal et al. 2011). However, for this action to occur, the peptides must first be resistant to amino- and carboxypeptidases in the gut epithelial cells, and secondly, transported intact through the cellular membrane to the blood, before being carried through the BBB (Ganapathy & Miyauchi 2005). de Magistris et al. (2010) found that there is a link between intestinal permeability and patients with autism. This can be explained by the "leaky gut" hypothesis; a condition where the intestinal wall is damaged, and peptides (e.g. BCM7) therefore could be transported more easily thorough the intestinal wall. This action is also dependent on the presence of the enzyme dipeptidyl peptidase-4 (DPP4). This enzyme can degrade the peptide before it is transported. Individuals with low DPP4 concentration may, therefore, be more vulnerable to BCM7. Dipeptidyl peptidase-4 is an enzyme that is mainly expressed on the surface of T-lymphocytes. Its foremost function is the inactivation of BAPs by removing the N-terminal dipeptide with P or A at the penultimate position of the peptide (Barnett et al. 2014). Studies have shown that individuals with lower DPP4 levels are more prone to the potential adverse effects of BCM7 (Clarke & Trivedi 2014).

1.6 Simulating Digestion of Food: A Static Method

There is a need for a standardized method for digesting food *in vitro* to simplify the technical challenges and minimize the experimental variables, so that experiments can be reproducible. The COST Action INFOGEST protocol (Minekus et al. 2014) has standardized an international method of *in vitro* digestion. The method simulates human gastrointestinal conditions. Furthermore, different parameters, such as time, temperature and use of enzyme

are described. The method has been validated in three inter-laboratory studies, where some parameters concerning protein digestion have been clarified (Egger et al. 2015). These studies also showed that the consensus method has led to an increased consistency and better comparability of *in vitro* digestion studies.

A static *in vitro* method have some disadvantages as compared to *in vivo* methods. The fixed parameters, such as time and pH, my limit some outcomes. However, the disadvantages of *in vivo* methods, concerning inter-individual variations and lack of reference standards, can make experiments difficult to compare and reproduce. In the present study, the use of human gastrointestinal juices will substitute the commercial enzymes, as stated in the INFOGEST protocol. The advantage of using human juices is that various isoforms of the enzymes are present and more likely to mimic an *in vivo* digestion. Therefore, the method used in this study is referred to as an *ex vivo* digestion model.

1.7 Protein Degradation Profile

1.7.1 Electrophoresis

For evaluation of the extent of digestion, it is desirable to identify proteins and peptides in the digests. Electrophoresis is a generic technique concerning the methods used for separation of molecules based on their size and charge in an electric field. Molecules such as proteins carry a net charge. However, when an anionic detergent is added to a protein sample, it applies a negative charge to the protein proportional to its mass. A common anionic detergent is sodium dodecyl sulphate (SDS), which breaks down the quaternary-, non-disulfid-linked tertiary structure as well as secondary structure of proteins. The intrinsic charge of the protein becomes insignificant when bound to SDS molecules, and therefore the unfolded polypeptide's charge and length will be proportional to the number of amino acid residues in the chain. The peptides will then migrate in the gel electrophoresis with relative mobilities depending only on their mass (Mathews et al. 2013). For further denaturation of protein structure, such as reducing disulfid linkages, a reducing agent is added and sample is heated to near boiling temperature. β-mercaptoethanol (BME) is a commonly used reducing agent for SDS sample preparation, however there are some disadvantages of using this agent. β-mercaptoethanol is volatile and may evaporate from solution. This may cause the concentration in solution to decrease and drive the equilibrium reaction to the left causing

more formation of disulfide linkages than reduction. A less toxic and volatile reducing agent is dithiothreitol (DTT), which also alters the conformation of the proteins containing cystein by forming ring structures with internal disulfide bonds, resulting in a non-reversible reaction after denaturation.

Another common technique for separation of peptides and proteins is capillary electrophoresis (CE). This method works with high and efficient separation and requires low sample volume. It is, like other electrophoresis techniques, based on ions' mobility in an electric field. As the ions migrate through the capillary, their UV absorbance is detected by spectrophotometric measurements, often at 214 nm for absorbance of peptide bonds, or 280 nm for absorbance of aromatic amino acids. Consequently, molecules are detected in an elctropherogram based on their absorbance and migration time.

1.7.2 Degree of Hydrolysis

During gastrointestinal digestion the proteins are hydrolysed by gastric and pancreatic enzymes. Due to this it is desirable to compare the degree of hydrolyses of different digestion steps, to evaluate the degradation process. The degree of hydrolysis (DH) is proportional to peptide bonds broken in a protein, and is calculated as follows:

$$DH\ \% = \frac{h}{h_{tot}} x 100\ \%$$

Where h is the amount of hydrolysed peptide bonds and where h_{tot} is the total amount of peptide bonds present (Rutherfurd 2010). By measuring the DH it is possible to determine the breakdown of peptides and average length of peptides for each phase in the digestion. There are several methods described for measuring DH, however, there has not been presented a consensus as to the best method. In a review of commonly used methods for determining DH, Rutherfurd (2010) found that the different methods both had advantages and disadvantages, and it was difficult to compare the methods.

The most common method for determining DH is pH-stat, where the amount of base required by titration of the reaction to keep a constant pH has a direct relationship to peptide bonds hydrolysed (Adler-Nissen 1986). Years before a method for determination of DH by

trinitrobenzenesulfonic acid (TNBS) was described by Satake et al. (1960). This method, as well as the o-phthaldialdehyde (OPA) method, is based on spectrophotometric assay and measured by absorbance at 340nm or with fluorometric readings (OPA). In the present study the determination of DH was done by fluorescamine assay described by Udenfriend et al. (1972). Fluorescamine (Fluram) binds to the primary amine in the side chains of peptides in the solution and becomes fluorescent, and is then observed spectrophotometric with excitation wavelength at 390 nm and an emission wavelength at 480 nm.

1.8 Peptide Profiling

There are different methods of peptide identification of hydrolysates after GI digestion. Within the proteomic field the use mass spectrometry (MS) has become the most efficient and accurate way of identifying complex mixtures of peptides. MS alone can provide molecular mass information of peptides with accuracy better than 0.01 %, depending on the ionization technique (Carr & Annan 2001). Additionally, when coupled to other methods (tandem mass spectrometry), MS can separate and identify peptides by their amino acid sequence. The technique for detecting molecular masses of peptides is based on the formation of gas-phase ions. Ions differ in their mass to charge *m/z* ratio, and can subsequently be separated in an electric field.

1.8.1 Separation of Peptides by Liquid Chromatography

The most common technique for analysing samples of complex mixture of peptides is coupling liquid chromatography (LC) to tandem MS. In mixtures where some peptide or proteins have a higher concentration than others, these species may dominate and mask others in the MS. The use of LC eliminates the need to fractionate complex mixtures prior to MS. A common method for separation is reversed-phase high performance LC (RP-HPLC). This technique is based on the charge, size and affinity of the analyte as it moves with the mobile phase through the stationary phase. If the analyte has a higher affinity to the stationary phase, the surface of the column, it will bind to the column and move slower than compounds with higher affinity to the mobile phase. Reversed-phase chromatography is based on a polar mobile phase and a hydrophobic stationary phase. As a result, hydrophobic molecules have a higher affinity to the stationary phase, and hydrophilic molecules are eluted

first through the column. When the LC is coupled to MS with an electrospray ionization inlet, the liquid analyte evaporates and becomes a gas, which is then analysed in the MS.

1.8.2 Formation of Gas-phase Ions by Electrospray Ionization

The gas-phase ions are formed from proteins and peptides by spraying a dilute solution of the analyte at atmospheric pressure from the tip of a fine stainless steel capillary (Carr & Annan 2001). The high voltage at the spray tip or counterplate causes the formation of fine droplets as the molecules get charged. The droplets evaporate and the molecules inside pick up one or more protons to form singly or multiply charged ions. As the droplets continue to shrink, the charge repulsion results in separation of the individual ions.

1.8.3 Tandem Mass Spectrometry

The tandem mass spectrometry (MS/MS) is based on steps of separation of ions. First, the precursor ion gets isolated and fragmented; secondly, the product ion is detected in the mass analyser. It is faster and more accurate than older techniques (e.g. Edman degradation). It is also able to identify peptides in mixtures and produce sequence information of modified peptides (Carr & Annan 2001). Individual charged ions enter into the quadrupole ion-trap. For further fragmentation of peptides the precursor ions undergo a collision induced dissociation, which results in peptide fragments a, b and c from N-terminal and x, y and z from C-terminal (Soares et al. 2012). The fragment ions produced are transmitted into the mass analyser and detected based on their *m/z* ratio. Figure 6 illustrates the principle of tandem MS.

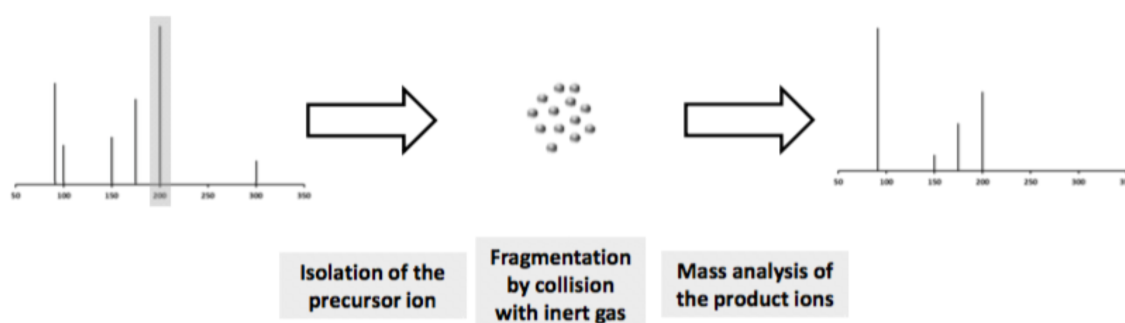

Figure 6. The principle of MS/MS ion fragmentation: The precursor ion is isolated in the first mass selector, then it undergoes fragmentation by collision in the ion-trap before the product ions are analysed in the second mass spectrometer. Adapted from Soares et al. (2012).

1.9 Aim of Thesis

Bioactive peptides are now considered dietary ingredients that may promote human health. On the other hand, some may also be involved in the development of certain diseases in humans. It has been hypothesised that BCM7 formed during digestion of different variants of β-CN can lead to the development of non-communicable diseases such as cancer, cardiovascular diseases, insulin-dependent diabetes and autism. Milk is a source of bioactive peptides and a possible precursor of BCM7; consequently, this nutrient laid the basis of the present study. Several *in vitro* digestion models have been approached in previous studies for evaluating the release of BCM7 during GI digestion of bovine β-CN and dairy products; however, the use of human gastrointestinal juice has not been applied for digestion of bovine milk and purified β-CN of different genetic variants, with subsequent identification of bioactive peptides. Consequently, the aim of this study was to investigate differences in peptides released from purified β-CN and bovine milk with different genetic β-CN variants (A1, A2, F and I) after *ex vivo* GI digestion, with particular interest in the release of β-casomorphins, BCM7 and others. An overview of the study design is presented in Figure 7.

The main aims of the study were to evaluate whether:

- Different genetic variants of bovine β-casein give rise to different peptides after GI digestion with human GI enzymes.
- Different amino acid substitutions in the protein sequence are responsible for different enzymatic cleavage sites during digestion.
- The use of *in vitro* digestion model with human gastrointestinal juices is more similar to *in vivo* digestion than using commercial enzymes of non-derived human sources.

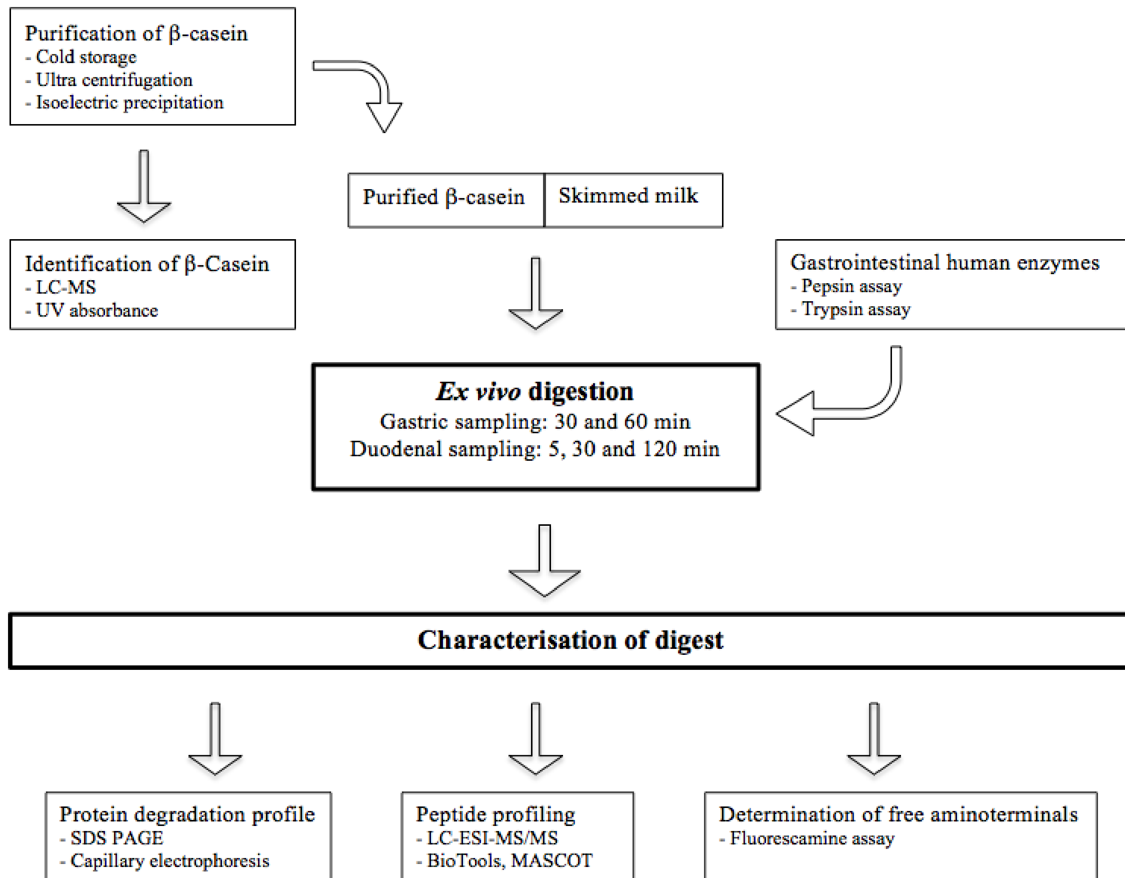

Figure 7. Overview of the study design

2 Materials and Methods

2.1 Isolation and Purification of β-casein Variants

The method for purification of β-casein was adapted from Petrat-Melin et al. (2015). β-casein was purified from skimmed milk from homozygous cows with identified genetic variants A1A1, A2A2, FF and II of the protein. After milking the milk was immediately frozen and stored at -20 °C. Prior to the experiment, the milk was thawed at 4 °C for 48 h and stirred for the last 24 h. For the separation of β-CN, the milk was ultracentrifuged at 38100 rpm (150 000 x *g*) at 4 °C for 2 h, using Optima L-80XP Ultracentrifuge (Beckman Coulter Inc., CA) with a titanium fixed-angle 70-Ti Rotor (angle 23°). The β-CN, in the supernatant, was then precipitated by lowering the pH to near its isoelectric point (4.8). To reach this pH, 10 % acetic acid was added to the amount of one-tenth of the final volume of the solution. When precipitation was visible, one-tenth of the final volume of 1 *M* sodium acetate was added to stabilize the pH. The samples were centrifuged at 1000 x *g* at 4 °C for 10 min in a Heraeus Multifuge 3 S-R centrifuge (Thermo Scientific), to recover the β-CN. The precipitate was washed three times with 10 mL MilliQ H_2O to remove the acetic acid. The β-CN was resuspended in MilliQ H_2O and snap frozen in liquid nitrogen. The isolated samples were then lyophilized and stored at -80 °C until further use.

2.2 Identification of Protein Variant by Liquid Chromatography – Mass Spectrometry

Lyophilized β-CN fractions were analysed to confirm the identity and purity of the different variants. The method was based on previous studies by Petrat-Melin et al. (2015), Jensen et al. (2012) and Frederiksen et al. (2011). Lyophilized β-CN fractions were dissolved in a solution containing 6 *M* guanidine hydrochloride (GndHCl) and 100 *mM* Bis-Tris to achieve a β-CN concentration of 10 mg/mL. 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 proteins were separated using an HPLC system with a Jupiter C4 column (300 Å pores, Phenomenex, US), at 40 °C, coupled to an 1100 series single-quadropole mass spectrometer (Agilent Technologies, US). The software ChemStation was used for identifying the individual protein peaks in the chromatogram. Integrated peak area was used to calculate the relative protein

content of the total integrated peak area in each chromatogram. All β-CN variants were analysed in duplicates.

2.3 Determination of Protein Purity of β-casein Variants by UV Absorption and Molecular Extinction Coefficient

The protein content of the purified β-CN fractions was determined by UV absorption, as previously described by Petrat-Melin et al. (2015). Lyophilized β-CN fractions were dissolved in 6 *M* GndHCl and 100 *mM* Bis-Tris to a final concentration of 1 mg/mL. 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 protein. The method was based on earlier work by Edelhoch (1967), that described the determination of molecular extinction coefficients at 280 nm (ε280) of Trp, Tyr and Cys, with the following equation:

$$Predicted\ Abs(\varepsilon 280) = \frac{\left(nTrp_{(i)}\ x\ 5500\right) + \left(nTyr_{(i)}\ x\ 1490\right) + \left(nCys_{(i)}\ x\ 125\right)}{MW_{(i)}}\ x\ Frac_{(i)}$$

Where *n* is the number of each residue in the protein, *MW* is the molecular weight of the protein; *Frac* is the relative amount of protein in the sample and where *i* is α_s-CN, β-CN, κ-CN or whey proteins in the purified sample isolated from skimmed milk. The amount of the individual proteins calculated from integrated peak area from LC-MS was used to determine the absolute β-CN content of the purified samples:

$$\beta - casein\ purity\ (\%) = \frac{measured\ abs280\ in\ purified\ samples}{predicted\ abs280\ at\ 1mg/ml}\ x\ 100$$

2.4 *In Silico* digestion

In silico methods are based on computed trials for simulation of experiments. The *in silico* digestion was performed using PeptideCutter (Expasy 2016), for simulation of proteolytic cleavage of proteins. The program is based on underlying algorithms, which can simulate

cleavage of known proteins with known enzymes to reflect an *in vivo* or *in vitro* digestion. The amino acid sequence of β-CN variants A1, A2, F and I was entered in the program, and the enzymes pepsin, trypsin and chymotrypsin (with low specificity) were selected to perform the simulated gastrointestinal digestion. The computer software predicted potential cleavage sites in the respective protein sequences.

2.5 *Ex Vivo* Gastrointestinal Digestion of Purified β-CN Variants and Skimmed Milk

2.5.1 Human Gastric and Duodenal Juices

Human gastric and duodenal juices were collected according to Ulleberg et al. (2011) and approved by the Norwegian Ethics Committee. Aspiration of 20 healthy volunteers from age 20 to 42 was performed at Moss Hospital, Norway. The volunteers were fasting for at least 8h 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. The pepsin and trypsin activities of the human gastrointestinal (GI) juices were assayed prior to the simulated digestion.

2.5.2 Gastrointestinal Digestive Enzyme Activities

The measurements and calculations of enzyme activities in the gastrointestinal juices were performed according to COST Action INFOGEST protocol (Minekus et al. 2014).

Pepsin Activity Assay

The principle of the assay is based on determination of TCA (trichloroacetic acid) soluble peptides released from haemoglobin by pepsin at pH 2 and 37 °C, measuring the absorbance at 280 nm of the TCA soluble hydrolysis products. The juice was diluted in 10 *mM* HCl to achieve different concentrations to ensure that the dose-response was linear. 2 % (w/v) Bovine haemoglobin was used as substrate adjusted to pH 2.0 with 300 *mM* HCl. The gastric juice was added to the substrate solution and incubated for exactly 10 min. The reaction was stopped by the addition of 5 % TCA. Blank samples were made for each diluted sample of the enzyme. All samples were centrifuged at 6000 rpm for 30 min, to precipitate the

unhydrolysed haemoglobin. The peptides in the supernatants were measured in a multi-mode microplate spectrophotometer (SpectraMax M2, Molecular Devices) with microtiter plates.

One unit of pepsin activity was defined as the amount of gastric juice (mL) that will produce an increase in A280 of 0.001 per min at pH 2.0 and 37 °C, measured as TCA soluble products.

The activity was calculated as follows:

$$\frac{Units}{mL} = \frac{[A280\ Sample - A280\ Blank]\ x\ 1000}{(\Delta t\ x\ X)}$$

Where Δt is the time (min) of the reaction and where X is the concentration (µg/mL) of the enzyme.

Trypsin Activity Assay

The principle of the assay is based on the determination of the hydrolysis product, p-toluene-sulfonyl-L-arginine, measured by a continuously reading of the absorbance at 247 nm. 10 *mM* p-toluene-sulfonyl-L-arginine methyl ester (TAME) was used as substrate and mixed with Tris-HCl buffer (0.046 *M* Tris, 0.0115 *M* $CaCl_2$ and 1 *M* HCl), pH 8.1. The reference sample was incubated in the spectrophotometer for 3 min to equilibrate the temperature 25 °C. Different concentrations of duodenal juices (5 µL–50 µL) were tested to ensure that the dose-response was linear. The absorbance was measured every 30 sec at 247 nm for 10 min in a multi-mode microplate spectrophotometer (SpectraMax M2, Molecular Devices).

One unit of trypsin activity was defined as the amount of duodenal juices (mL) that hydrolyses 1 µmole of TAME per min at 25 °C and pH 8.1.

The activity was calculated as follows, using the maximum linear rate and over at least 5 minutes:

$$\frac{Units}{mL} = \frac{[(\Delta A247\ Sample - \Delta A247\ Blank)\ x\ 1000\ x\ V]}{540\ x\ X}$$

Where $\Delta A247$ is the linear portion of the curve (unit abs/min), V is the total volume (mL) of sample, 540 is the molar extinction coefficient of TAME at 247 nm, and X is the amount of trypsin (mL).

2.5.3 *Ex Vivo* Digestion Model

Model digestion with human GI enzymes was performed according to the COST Action INFOGEST protocol (Minekus et al. 2014), with some modifications. The method is illustrated in Figure 8. Due to the use of human enzymes, the volume was adjusted to one-tenth of the considered volume in the INFOGEST protocol. The oral phase was not included due to the digestion of milk (no chewing and no amylase activity needed).

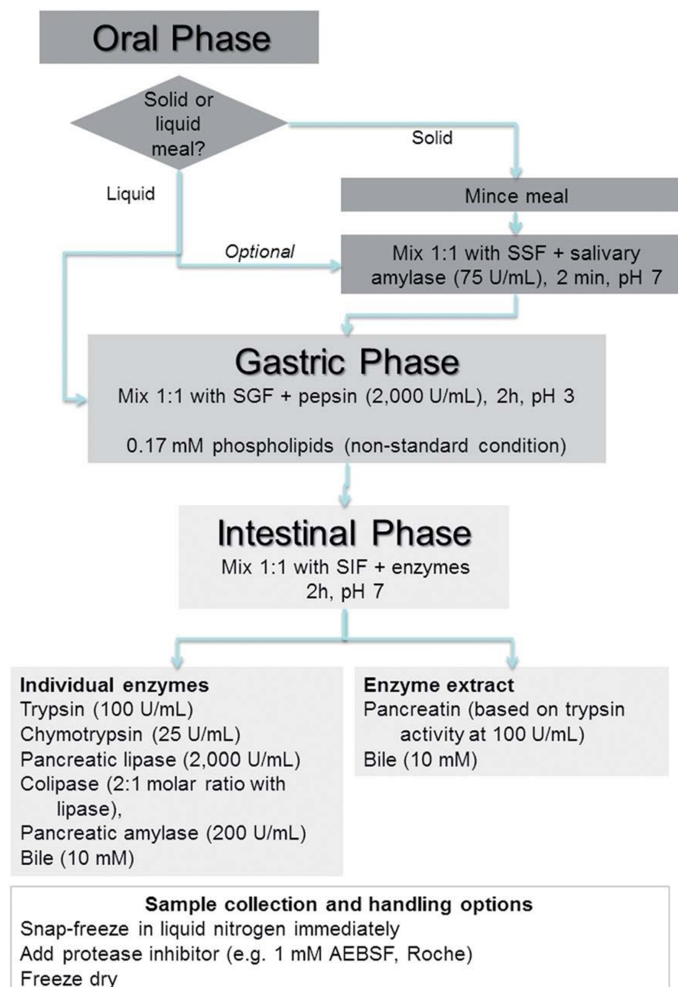

Figure 8. Flow diagram of simulated digestion adapted from COST Action INFOGEST (Minekus et al. 2014).

The digestion was carried out with 1 mL of skimmed milk or 1 mL of purified β-CN. The purified β-CN was diluted in simulated milk ultrafiltrate (SMUF; (Jenness & Koops 1962) to 10 mg/mL prior to digestion. The milk or purified β-CN was mixed 50:50 (v/v) with simulated gastric fluid (SGF) (components listed in Table 3), and human gastric juices (HGJ) (2000 U/mL). pH was adjusted to 3.0 by adding 1 *M* HCl. The samples were incubated in a waterbath at 37 °C with magnetic stirring. After 60 min of gastric digestion, simulated intestinal fluid (SIF; Table 3) and human duodenal juices (HDJ, 100 U/mL) was added to the gastric sample 50:50 (v/v). pH was adjusted to 7.0 by addition of 1 *M* NaOH, which effectively abolishes pepsin activity. The samples were incubated at 37 °C for 120 min. Sampling was done after the gastric phase at 30 min (only milk samples) and 60 min. Inactivation of the pepsin activity was done by increasing the pH > 6 with 1 *M* $NaHCO_3$ and cooling directly on ice. Sampling in the intestinal phase was done at 5, 30 (only milk samples) and 120 min by adding 5*mM* Pefabloc® (76307) (Sigma Aldrich) to the samples for enzyme inactivation and cooling on ice. All samples were performed in duplicates and immediately frozen at -20 °C, and stored until further use.

Table 3. Electrolyte stock solution prepared for gastric and duodenal phase of digestion (Minekus et al. 2014).

Salt solution added	**Stock concentration**		**Amount for 0.4L (1,25x)**	**Final conc. in SGF**	**Amount for 0.4L (1,25x)**	**Final conc. in SIF**
			SGF (pH 3)		SIF (pH 7)	
	g/L	mol/L	mL	mmol/L	mL	mmol/L
KCl	37.3	0.5	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	0.9	0.9	0.8	0.8
$NaHCO_3$	84	1	12.5	25	42.5	85
NaCl	117	2	11.8	47.2	9.6	38.4
$MgCl_2(H2O)_6$	30.5	0.15	0.4	0.12	1.1	0.33
$(NH4)_2CO_3$	48	0.5	0.5	0.5	-	-
$CaCl_2(H2O)_2$	44.1	0.3	-	0.15	-	0.6
HCl	-	6	1.3	15.6	0.7	8.4

2.6 Protein Degradation Profile by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The samples were analysed by SDS-PAGE to observe the enzymatic degradation of proteins during the digestion. All samples, undigested and digested, were diluted to the same concentration (≈4 mg/mL) before being mixed 1:1 with fresh SDS buffer containing 5 *mM* dithiothreitol (DTT) and heated at 95 °C for 5 min. 10 µL of sample was added to each well of a 10 % Mini-PROTEAN TGX Stain-Free Precast Gel (Bio-Rad), and run at 200 V for 25 min. Low molecular weight protein ladder was used as a standard. The proteins were fixed and stained with Comassie Brilliant Blue (Bio-Rad), then de-stained and kept in preservation solution (10 % glycerol, 10 % CH_3OH).

2.7 Determination of Free Amino Terminals of the peptides by Fluorescamine Assay

The purpose of this method was to determine the peptide concentration by measuring free amino terminals that represent the number of peptide bonds that has been hydrolysed and determined as degree of hydrolysis (DH). The method has previously been described by Larsen et al. (2004). Digested samples were reduced and alkylated to achieve a cleaner sample prior to the assay: Ammonium bicarbonate (0.05 *M*) was added to the digested samples to a final volume of 100 µL and protein concentration of 1 mg/mL. Reduction was achieved by adding 10 µL of DTE (10 mg/mL in 0.05 *M* NH_4HCO_3) to the solution, followed by incubation at 60 °C for 10 min. For alkylation, 10 µL of Iodoacetamide (IA) (50 mg/mL in 0.05 *M* NH_4HCO_3) was added, and the solution was incubated in the dark at 37 °C for 30 min. 40 µL of formic acid (6.25 %) was added to acidify the sample. The solution was filtered through a 10 kDa molecular weight cut-off filter by centrifugation at 14000 x *g* and at 4 °C for 10 min. Samples can also be precipitated by TCA according to Petrat-Melin (2014). After reduction and alkylation 30 µL digested sample was mixed with 900 µL 0,1 *M* Na-borat buffer (pH 8,0) and 300 µL Fluram® (Sigma Aldrich) (0,2 mg/mL in dried acetone). A standard curve was prepared with concentrations of Leucine at 0.50, 0.75, 1.0, 1.5, 2.0 and 3.0 *mM*. Human gastrointestinal juices were used as blank samples. Fluorescent compounds were detected with an excitation wavelength at 390 nm and an emission wavelength at 480 nm. The DH was calculated as follows:

$$DH\ \% = \frac{[-NH_2]_d - [-NH_2]_0}{[-NH_2]_\infty - [-NH_2]_0} x100$$

Where [-NH_2] is the concentration of primary amines in the digested (d) or undigested (0) samples calculated as leucine equivalents read from the standard curve, and $[-NH_2]_\infty$ is the theoretical maximal primary amine concentration assuming total digestion of free amino acids, and is calculated as follows:

$$[-NH]_\infty = \frac{(1 + f_{lys}) * C_{CN}}{MW_{AA}}$$

Where f_{lys} is the fraction of Lysine residues in the casein, C_{CN} is the casein concentration in the sample, and where MW_{AA} is the mean molecular weight of amino acids in the casein.

2.8 Protein Quantification and Identification by Capillary Electrophoresis (CE)

Protein and peptide quantification by CE was performed on undigested samples and digested samples from the gastric phase. The human gastric enzymes were also analysed to quantify any interference of the absorbance in the digested samples. The method was performed according to Heck et al. (2008) and Mestawet et al. (2014) with some modifications. All samples were mixed with sample buffer containing 6 *M* urea, 0.83 mg/mL hydroxypropyl methyl cellulose (HPMC), 42 *mM* 3-morpholinopropanesulphonic acid (MOPS), 167 *mM* Tris, 67 *mM* ethylenediamine tetra acetic acid disodium salt dehydrate (EDTA) and 7.9 mg/mL DTT (pH 8.6). All samples were stirred and put on an orbital shaker for 1 h before centrifugation at 5000 *g* in room temperature for 5 min. The samples were filtered through a 0.45 µm polyethersulfone membrane filter (Millex, Carrigtwohill, Ireland) with a 25 mm syringe (BD Plastipak™ VWR International, USA) and transferred to CE sample vials (50 µL) (Agilent Technologies). CE analyses was performed on Agilent Capillary electrophoresis system controlled by Agilent ChemStation software (version B.03.02(341)) (Agilent Technology, Germany) and coupled to a Minichiller (Huber, Germany) set at 20 °C for temperature equilibrium. Separation was performed using CE standard bare fused-silica capillary (Agilent Technologies) with dimensions 50 µm i.d. and 56 cm. Separation was carried out at 45 °C and a linear voltage gradient from 0 to 25 kV in 3 min was used, followed by constant voltage at 25 kV. All samples were injected at a pressure of 34.5 mbar

for 20 sec and the UV detection was performed at 214 nm. The capillary was flushed with 0.1 *M* NaOH for 5 min, followed by flushing with run buffer (6 *M* urea, 0.83 mg/mL HPMC, 20 *mM* sodium citrate, 0.19 *M* citric acid, pH 3.0) for 20 min. Integrated peak area was used to determine the degree of digestion of the milk proteins. The identification of the different genetic variants of β-CN was performed according to Heck et al. (2008). The area under the graph for each peak was integrated and used to determine the degree of digestion, and the relative amount of casein in purified samples.

2.9 Peptide Profiling by Liquid Chromatography – Electrospray Ionisation Tandem Mass Spectrometry (LC-ESI-MS/MS)

Peptide identification by LC-ESI-MS/MS was done according to Petrat-Melin (2014) with some modifications. To minimize unwanted interferences of the MS analyses, the digested samples were reduced, alkylated, and filtered using a 10 kDa cut-off spin-filter (Millipore, Cork, Ireland) to remove undigested protein and proteases, as described in chapter 2.7. All samples were diluted 3x in 0.1 % formic acid prior to LC-MS/MS analyses. The peptides were separated using an LC-system consisting of a 1200 series capillary pump and autosampler (Agilent Technologies, Waldbronn, DE) fitted with a Jupiter C18 300-Å micro-column (Phenomenex, Værløse, DK) (with dimensions 150 mm x 0.5 mm and particle size of 5 µm), operated at 20 °C, and coupled to an ESI-ion-trap mass spectrometer (Bruker Daltonik, Bremen, DE). The LC gradient, consisting of solvent A (0.1% formic acid) and B (90 % acetronitrile, 0.1 % formic acid), was set at 2 % B for 10 min, 40 % B for 70 min, 80 % B for 15 min and decreasing to 2 % for 15 min, with flow rate at 200 µL/min. MS scans were conducted on peptides from a m/z 50 to 3000. Sample injection volume was 5 µL and 10 µL for purified β-CN and milk, respectively. Mascot database (Matrix Science, MA, US) recorded the spectra and searched for known genetic variants of bovine milk proteins with no specific enzyme cleavage sites, mass tolerance of 0.5 % and MS/MS mass tolerance of 0.5 Da. DataAnalysis (version 4.0) and BioTools (version 3.1) (Bruker Daltonik, Bremen, DE) were used to process the MS/MS spectra and to identify peptides with a mascot score lower than the significance threshold ($p < 0.05$).

2.10 Statistical Analysis

Purification of β-CN variants was performed from one milk sample from one homozygous cow, and the identity of each variant by LC-MS was performed in duplicates. The average area in the chromatogram of the duplicates for each variant was used to determine the β-CN purity together with quadruplicates of measured absorbance (280 nm). The digestion was performed in two independent experiments following assays of the duplicates (SDS-PAGE and degree of hydrolysis). Peptide analyses, Mascot search and capillary electrophoresis were performed with one of the two independent experiments from the digestion. Two different control samples (human GI enzymes and undigested milk/β-CN) were prepared for subtracting values in the digested samples. Fluorescamine assay was performed in triplicates. The statistical software package R (version R 3.2.3) was used to determine differences in the degree of hydrolysis after digestion by two-way ANOVA. Genetic variant and digestion time were used as factors in the ANOVA. To indicate statistically significant differences p-values < 0.05 were chosen.

3 Results

3.1 Identity and Purity of β-CN

β-casein from milk homozygous for the genetic variants A1, A2, B, F and I was isolated by the process of cold storage, ultracentrifugation and isoelectric precipitation. Chromatograms from LC-MS provided the molecular weight of the proteins in the purified samples, which enabled the identification of the β-CN variants. The integrated area under the graph for each peak was used to determine the purity of the isolated CN, together with measured and predicted absorbance at 280 nm.

Lyophilized β-CN samples were weighted after purification. Considering the relative amount of protein in milk, and assuming 40 % of the CN is β-CN, the yield from purification of β-CN was calculated by the amount (mg) obtained from the milk sample. The yield was estimated to be between 19 % and 35 %. The highest yield (35 %) was obtained from the I variant and the lowest yield (19 %) was obtained from the A1 variant.

3.1.1 Molecular Mass of Purified β-CN Variants

The molecular mass of the isolates was determined by LC-MS analysis. This confirmed the identity and homozygosity of the purified β-CN variants. However, one milk sample claimed to be homozygous for the B variant was identified homozygous for the A2 variant after mass analysis. Therefore, this sample is not further considered in the present study. The variant claimed to be homozygous for the F variant was later identified as the heterozygote A2F β-CN. This variant is referred to as A2F in chapter 3.3, and further. The chromatograms for each variant are illustrated in Figure 9 with the respective mass, measured with absorbance at 214 nm. The MS analysis of the peaks in the chromatograms representing β-CN showed molecular masses of 24 018 Da, 23 978 Da, 24 034 Da and 23 960 Da for variants A1, A2, F and I, respectively.

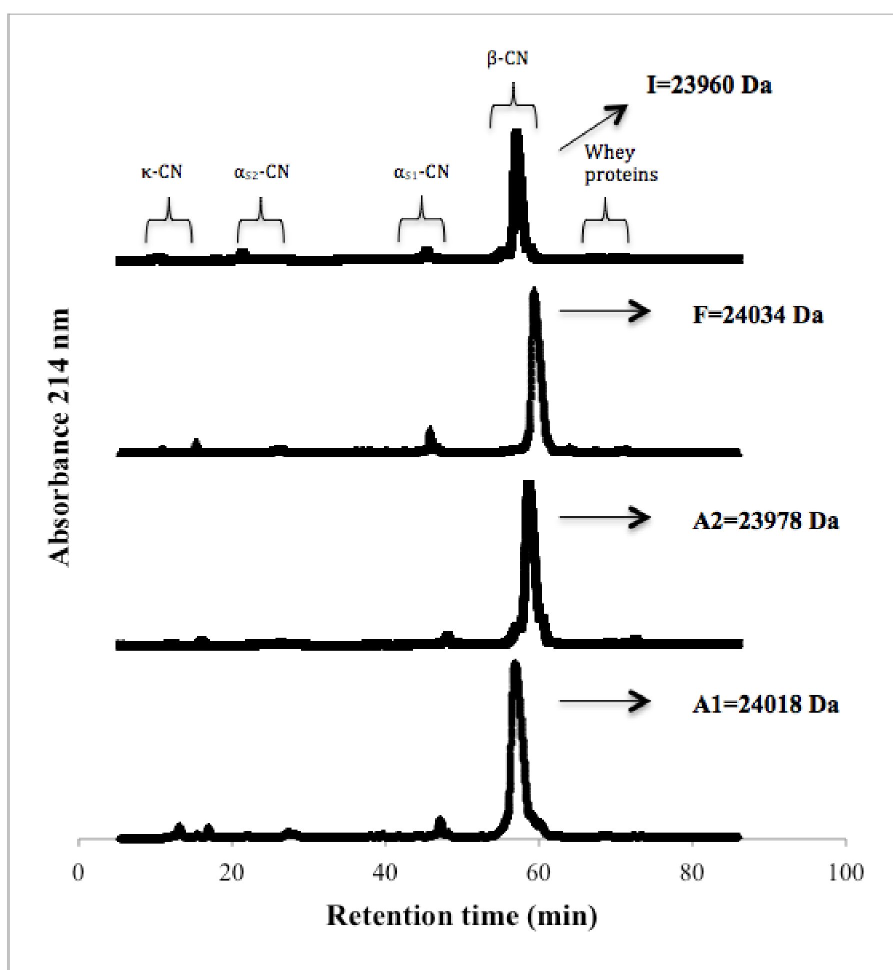

Figure 9. Chromatogram of purified β-CN variant A1, A2, F and I. Each β-CN variant is illustrated with a peak at its retention time and corresponding mass (dalton, Da). Minor peaks represent the other CNs and whey proteins in the samples. All samples were analysed in duplicates.

3.1.2 Analysis of Purity of β-CN Variants

The absorbance of the lyophilized β-CN variants was measured at 280 nm in order to compere the predicted and measured absorbance of the purified sample, to establish the absolute CN concentration. Each identified peak in the chromatogram determined the relative amount of the proteins, and the molecular extinction coefficient was used to determine the predicted absorbance at 280 nm of 1 mg/mL protein for each sample. The measured absorbance of each β-CN variant together with the predicted absorbance was used to calculate the purity of the purified β-CN:

$$\beta - casein\ purity\ (\%) = \frac{measured\ abs280\ in\ purified\ samples}{predicted\ abs280\ at\ 1mg/mL} x\ 100$$

The mean protein content of the duplicates of purified samples is presented in Table 4. The purity of β-CN relative to total protein in the purified samples varied from 82.7 % to 90.5 %. The second highest amount of protein in the samples was α_{S1}-CN, which constituted 4.25 % in average of the total protein amount. The other proteins varied from 0.4 % to 3.3 %. The purity of each β-CN variant was further used when determining the amount of β-CN needed for the *ex vivo* GI digestion.

Table 4. Mean protein content (%) of purified β-CN variants measured by relative peak area of the chromatogram by LC-MS and absorption at 280 nm. Values are expressed as mean percentage ± SD (n=2).

β-CN variant	**β-CN**	**κ-CN**	**α_{S1}-CN**	**α_{S2}-CN**	**Whey proteins**
A1	88.7 ± 1.03	1.2 ± 0.02	3.6 ± 0.14	1.3 ± 0.17	0.4 ± 0.19
A2	90.5 ± 1.04	0.8 ± 0.06	2.9 ± 0.18	1.6 ± 0.03	1.6 ± 0.39
F	82.7 ± 0.09	1.8 ± 0.01	6.3 ± 0.01	2.3 ± 0.32	0.5 ± 0.04
I	84.1 ± 0.15	2.2 ± 0.08	4.4 ± 0.01	1.2 ± 0.01	3.1 ± 0.03

3.2 *In Silico* Digestion

In silico digestion was performed using PeptideCutter (Expasy 2016), a computed digestion for simulating enzymatic hydrolysis of proteins. The proteolytic enzymes pepsin, chymotrypsin and trypsin were selected to reflect an *in vivo* or *in vitro* digestion. The results of digesting β-CN (genetic variant A1, A2, F and I) using PeptideCutter, is presented in Table 5. Only peptides in the region of polymorphism or peptides cleaved at the site of amino acid substitution is shown in the table.

The results of *in silico* gastric digestion with pepsin was identical for the A1 and A2 β-CN variants. The I variant generated two additional sites of cleavage by pepsin, at position 92 and 93, where the I variant contains a M93L substitution. The F variant generated one additional cleavage at position 152, which contains the P152L substitution. In the simulated duodenal digestion with chymotrypsin the results were similar among all genetic variants except the A1 and F, which resulted in an additional cleavage C-terminally of the polymorphic site at position 67 (P67H). The F variant also resulted in cleavage at position 151, N-terminal of the amino acid substitution (P152L). Otherwise, all results were equal among the variants and

there were no differences in the cleavage sites with trypsin. All results observed after simulated proteolytic cleavage with trypsin were fragments cleaved C-terminally to R and K, which is characteristic for this enzyme (MEROPS 2016).

Table 5. Peptide fragments resulting from *in silico* digestion of β-casein variants A1, A2, F and I, performed by the software PeptideCutter (Expasy 2016). The enzyme pepsin, chymotrypsin (low specificity) and trypsin were chosen for simulating gastrointestinal digestion. Only peptide fragments in the region of polymorphism are presented.

β-Casein variant	**Position [a]**	**Sequence [b]**	**Enzyme**
A2, I	61-70	PFPGPI**P**NSL	Pepsin
A1, F	61-67	PFPGPI**H**	Pepsin (N-term), Chymotrypsin (C-term)
I	89-92	QPEV	Chymotrypsin (N-term), Pepsin (C-term)
A1, A2, F	89-93	QPEV**M**	Chymotrypsin
A1, A2, I	149-156	QPL**P**PTVM	Chymotrypsin
F	149-151	QPL	Pepsin
F	153-156	PTVM	Pepsin (N-term), chymotrypsin (C-term)

[a] Position in the mature β-CN sequence, not including the signal sequence

[b] Amino acid sequence using one letter abbreviation. Bold letters denote site of amino acid substitution

3.3 *Ex Vivo* Gastrointestinal Digestion

3.3.1 Enzyme Activities in HGJ and HDJ

Pepsin activity in HGJ and trypsin activity in HDJ was measured prior to GI digestion. The pepsin activity was based on the determination of TCA soluble products produced by HGJ and calculated to 909 U/mL HGJ. The trypsin activity was determined by the hydrolysis of TAME by HDJ and was calculated to 21.9 U/mL.

3.3.2 SDS-PAGE Degradation Profile of Digested Proteins

The digested milk proteins and purified β-CN were separated to study the degradation patterns. The digested samples were run on a SDS-PAgel as illustrated in Figure 10. There were no major visible differences in the degradation profiles of the different genetic variants; thus, the figure represents all variants, here illustrated with variant A1. After initial 30 min of gastric digestion, the major fraction of each CN was digested. Furthermore, after 5 min of duodenal digestion, almost all proteins, including whey proteins, were completely digested. The degradation profiles also showed that the bovine serum albumin was digested in the gastric phase; however, the other whey proteins were not digested before initial 5 min of duodenal digestion. After digestion of purified β-CN, only small faint bands were visible. The bands visible around 30-60 kDa (a, b, c and d) represented the human duodenal enzymes, previously identified by Devle et al. (2014).

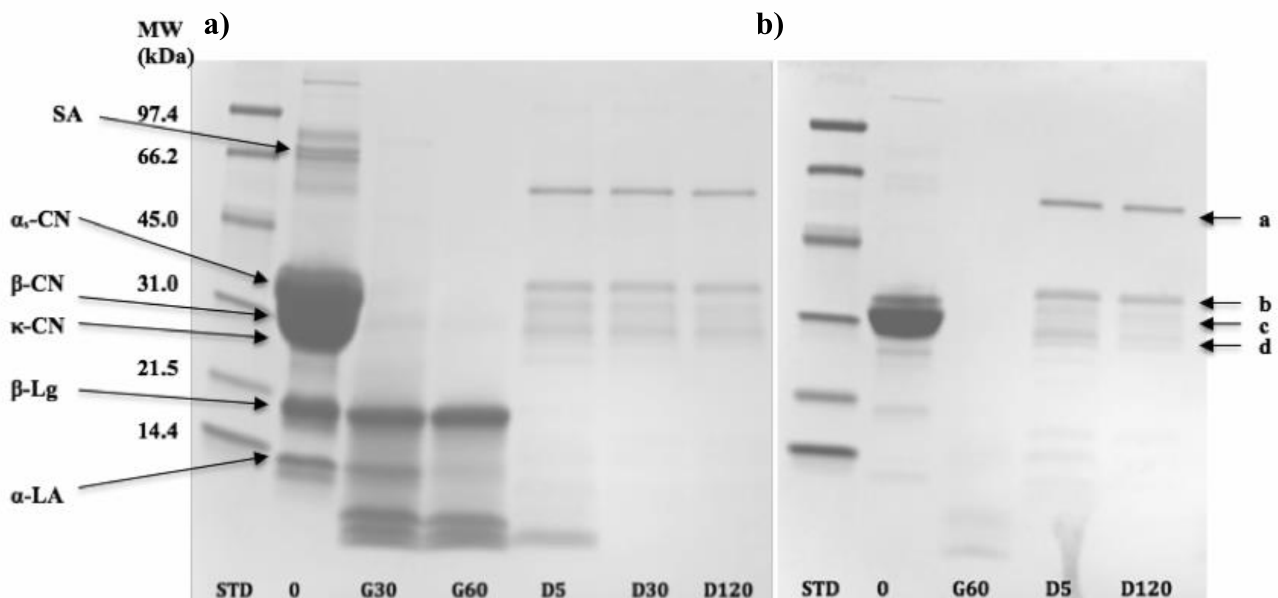

Figure 10. Protein degradation profile of a) skimmed milk (variant A2) and b) purified β-CN (variant A2) after *ex vivo* digestion: STD, low molecular weight standard; 0, undigested; G30 and G60, gastric digestion with HGJ for 30 min and 60 min, respectively; D5, D30 and D120, duodenal digestion with HDJ for 5, 30 and 120 min, respectively, after initial 60 min of gastric digestion; a, amylase; b, carboxypeptidase, chymotrypsin, elastase, lipase, gastricsin and amylase; c, carboxypeptidase, elastase, lipase, trypsin and amylase; d, carboxypeptidase, chymotrypsin, elastase, lipase, trypsin and amylase (enzymes previously reported by Devle et al. 2014).

3.3.3 Degree of Hydrolysis of Digested Proteins

Fluorescamine assay was performed to determine the degree of hydrolysis (DH) of the digested proteins. Degree of hydrolysis is the percentage of hydrolysed peptide bonds in the protein. This measurement can therefore estimate how much the protein has been degraded, and the average length of the peptides. The data is presented in Table 6 and Table 7. The DH of proteins in skimmed milk after gastric digestion varied from 6.8 % to 15.9 %, and increased to approx. 50 % after duodenal digestion. In the purified β-CN hydrolysates the DH in the gastric phase was measured to about 27 % and increased to approx. 73 % after 120 min duodenal digestion. The overall DH was considerably higher of purified β-CN samples than skimmed milk samples. Statistical analysis revealed that there were no significant differences ($p < 0.05$) in DH between the genetic variants of β-casein after digestion.

Table 6. Degree of hydrolysis (DH) of milk proteins after *ex vivo* digestion of skimmed milk. Values are expressed as mean percentage ± SD (n=6).

Variant	Gastric 30 min		Gastric 60 min		Duodenal 5 min		Duodenal 30 min		Duodenal 120 min	
A1	9.5	± 0.04	7.5	± 1.15	41.8	± 0.09	41.7	± 2.46	43.8	± 1.52
A2	15.9	± 4.63	12.6	± 0.42	51.3	± 0.21	59.6	± 2.81	65.2	± 3.63
A2F	14.6	± 1.71	14.6	± 0.17	45.4	± 0.32	51.9	± 3.19	54.2	± 5.47
I	6.8	± 1.79	11.5	± 6.29	55.1	± 0.15	69.5	± 3.19	41.1	± 7.50
Mean	**11.7**	**± 2.00**	**11.6**	**± 2.00**	**48.4**	**± 0.20**	**55.7**	**± 2.90**	**51.1**	**± 4.50**

Table 7. Degree of hydrolysis (DH) of purified β-CN after *ex vivo* digestion. Values are expressed as mean percentage ± SD (n=6).

Variant	Gastric 60 min		Duodenal 5 min		Duodenal 120 min	
A1	28.2	± 0.88	73.3	± 3.71	76.3	± 0.54
A2	31.3	± 0.25	75.1	± 4.38	60.3	± 7.40
A2F	31.6	± 1.67	80.4	± 0.17	83.4	± 15.73
I	19.5	± 8.34	77.6	± 8.28	72.5	± 12.68
Mean	**27.6**	**± 2.78**	**76.6**	**± 4.13**	**73.1**	**± 9.09**

Estimation of mean length of peptides (MLP) was performed for the hydrolysates. The DH of gastric digestion of skimmed milk revealed that 11 % of peptide bonds were broken. This is equal to MLP of 9 amino acids (100 %/DH). The MLP of the duodenal digestion of skimmed milk was estimated to 2 amino acids. Furthermore, the estimated MLP in the digestion of purified β-CN was 3.7 and 1.3 in the gastric and duodenal phase, respectively.

3.3.4 Capillary Electrophoresis

The degradation of proteins during gastric phase of digestion was evaluated by capillary electrophoresis. The electropherograms representing the undigested and digested samples of skimmed milk and purified β-CN with genetic variant A1, A2, A2F and I are presented in Figure 11 – Figure 15. The first 13 min is excluded from the electropherograms due to high interference peaks at 0-5 min and no visual absorbance from 5-13 min. The electropherograms in Figure 11 illustrates purified β-CN variants before and after 60 min of gastric digestion. There were no visual differences in the degradation profiles between the variants, however, the different genetic β-CN variants eluted at different times. β-casein A1 and A2F eluted earlier (at approx. 31 min) than the A2 and I variant (at approx. 32 min). The heterozygote β-CN A2F only contained one peak in the electropherogram corresponding to the F variant. After 60 min of gastric digestion the peaks representing β-CN were not visible and a few smaller unidentified peaks were detected. Furthermore, the area under the graph for peaks representing β-CN was calculated and compared to total area. The amount of β-CN in the purified samples was 70 %, 73 %, 74 % and 77 % for A2F, I, A1 and A2, respectively. Figure 12 illustrates one representative sample of the purified β-CNs. The electropherogram shows smaller peaks in the digested sample at 27 and 30 min, which could represent αS_1 and κ-CN, respectively. Other peaks observed in the digested sample represent peptides formed after digestion.

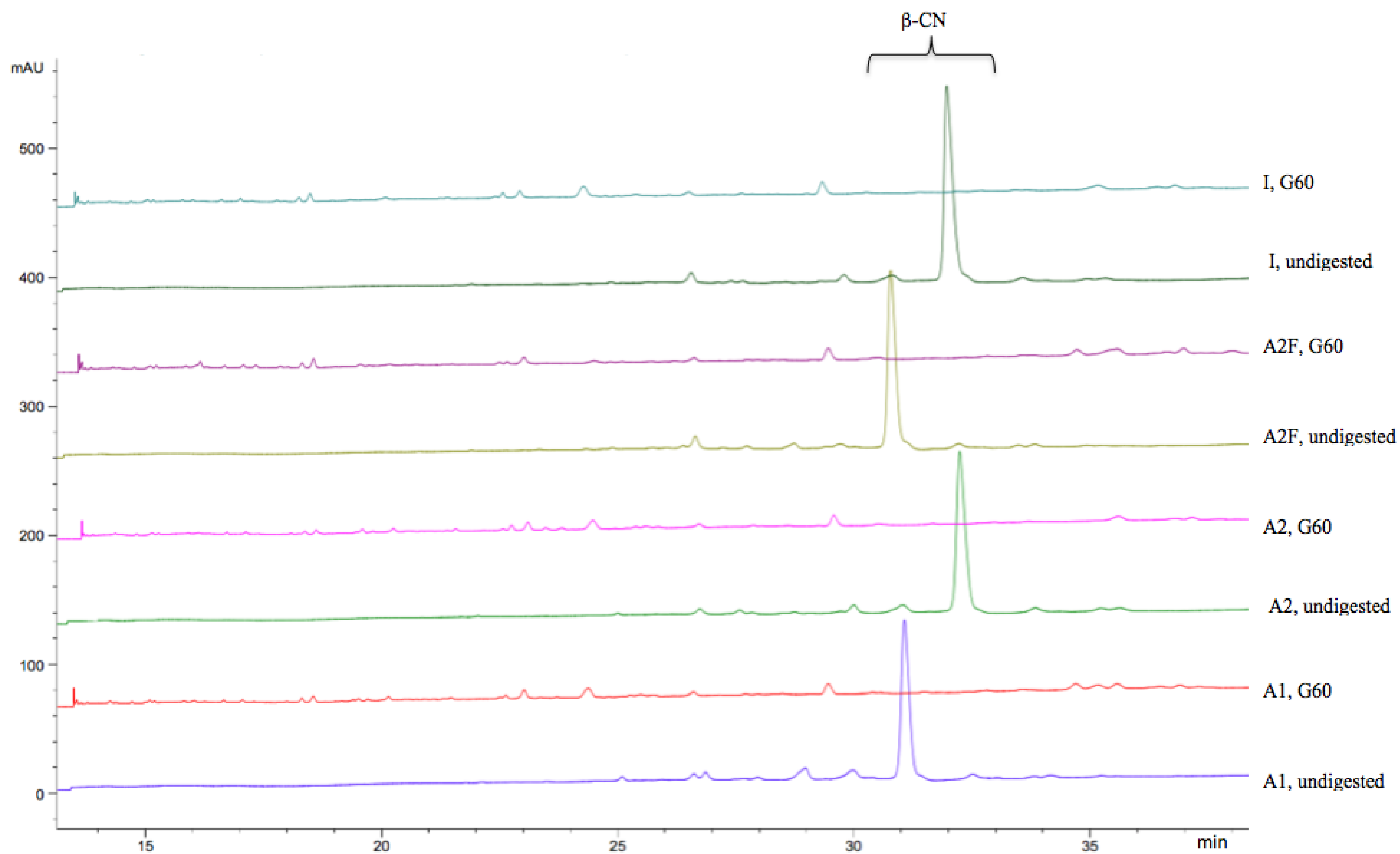

Figure 11. Electropherograms with absorbance at 214 nm of all undigested purified β-casein variants and variants digested 60 min in the gastric phase. The difference in concentration of the undigested and digested samples is 2:1.

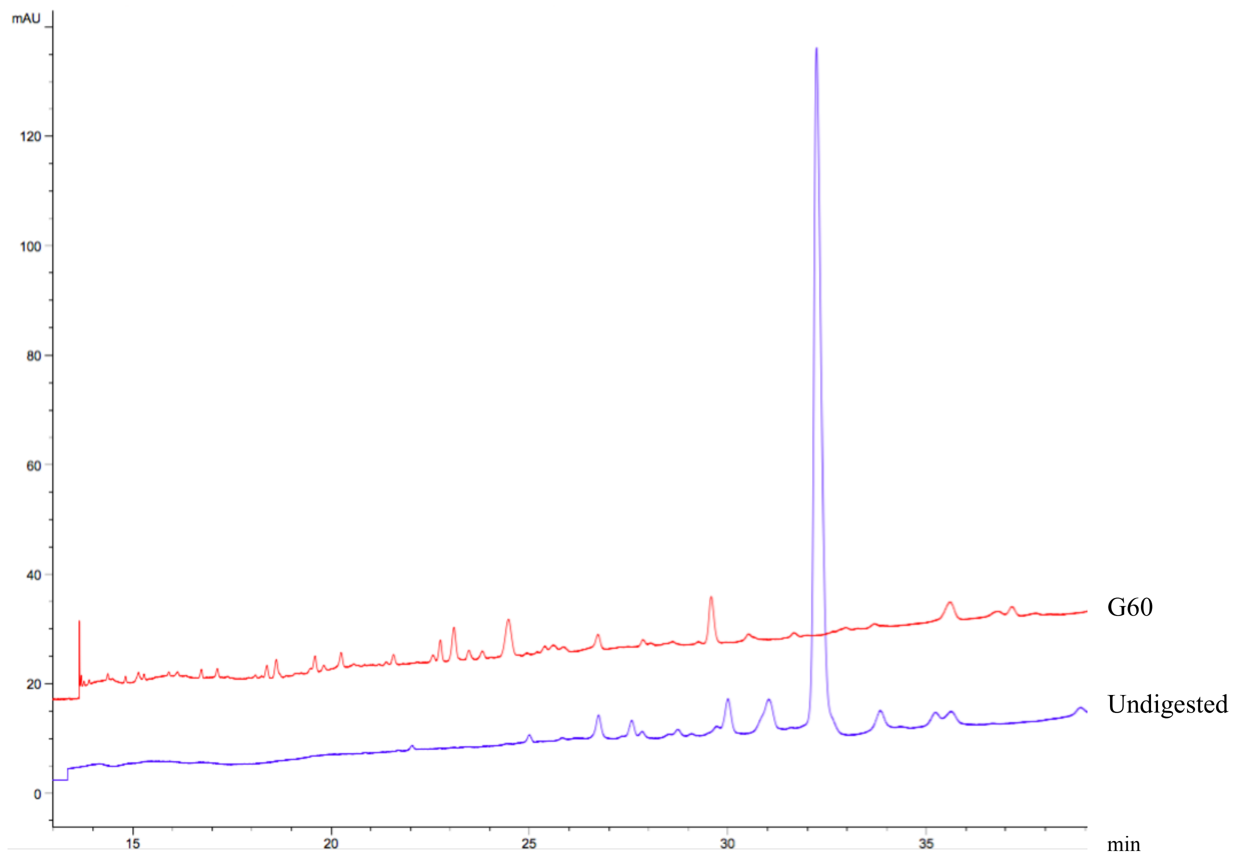

Figure 12. Electropherogram with absorbance at 214 nm of purified A2 β-casein; undigested and digested 60 min in the gastric phase. The difference in concentration of the undigested and digested samples is 2:1.

The electropherogram in Figure 13 illustrates the degradation of skimmed milk samples after 30 and 60 min of gastric digestion. The undigested samples were identified according to Heck et al. (2008), with peaks representing the milk proteins: α-lactalbumin (α-LA), β-lactoglobulin (β-LG), and the CNs αS_1, αS_2, κ and β. Looking more closely at the degradation profile in Figure 14, it is possible to suggest that the peaks identified at 22, 27, 30 and 33.5 min represents β-LG, αS1-CN, κ-CN and β-CN, respectively. Furthermore, to observe any differences in degradation pattern between the genetic variants, all milk samples from 30 min of gastric digestion were collated, as seen in Figure 15. The electropherogram showed some small differences at elution time 16 and 26-27 min, where the A2F sample differed most.

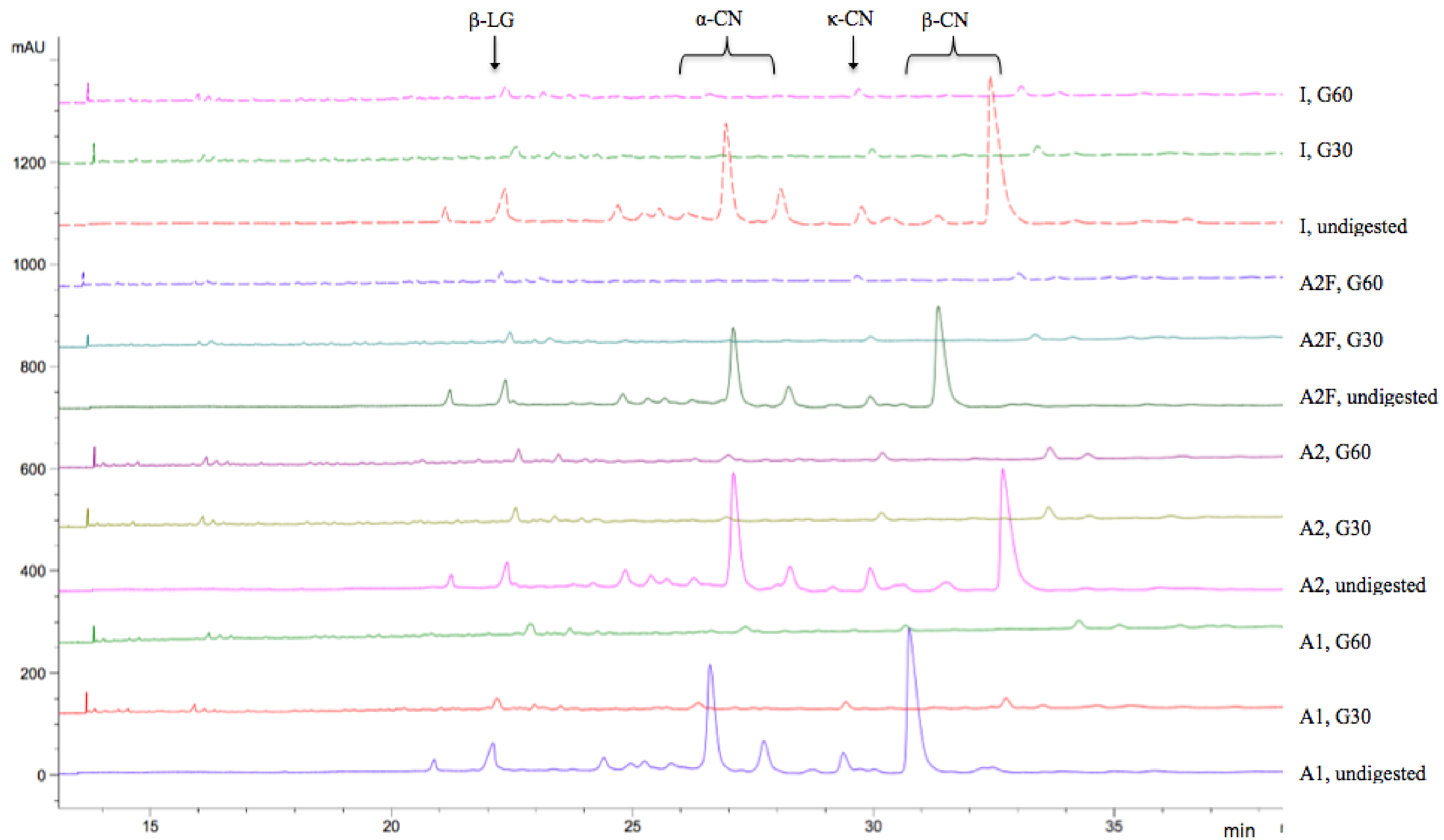

Figure 13. Electropherograms with absorbance at 214 nm of all undigested skimmed milk samples and samples digested 30 and 60 min in the gastric phase. The difference in concentration of the undigested and digested samples is 2:1.

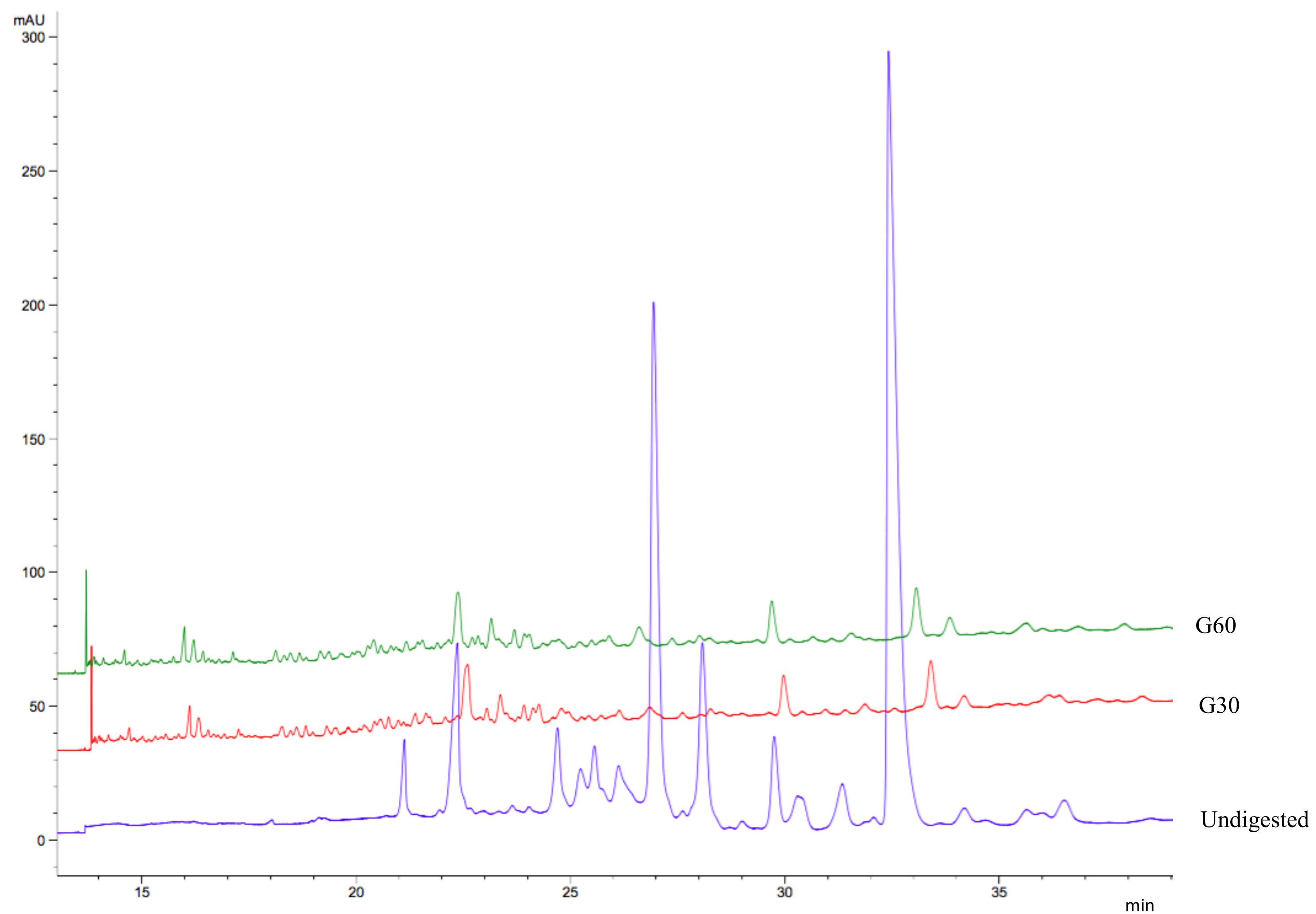

Figure 14. Electropherogram with absorbance at 214 nm of A2 skimmed milk; undigested and digested 30 and 60 min in the gastric phase. The difference in concentration of the undigested and digested samples is 2:1.

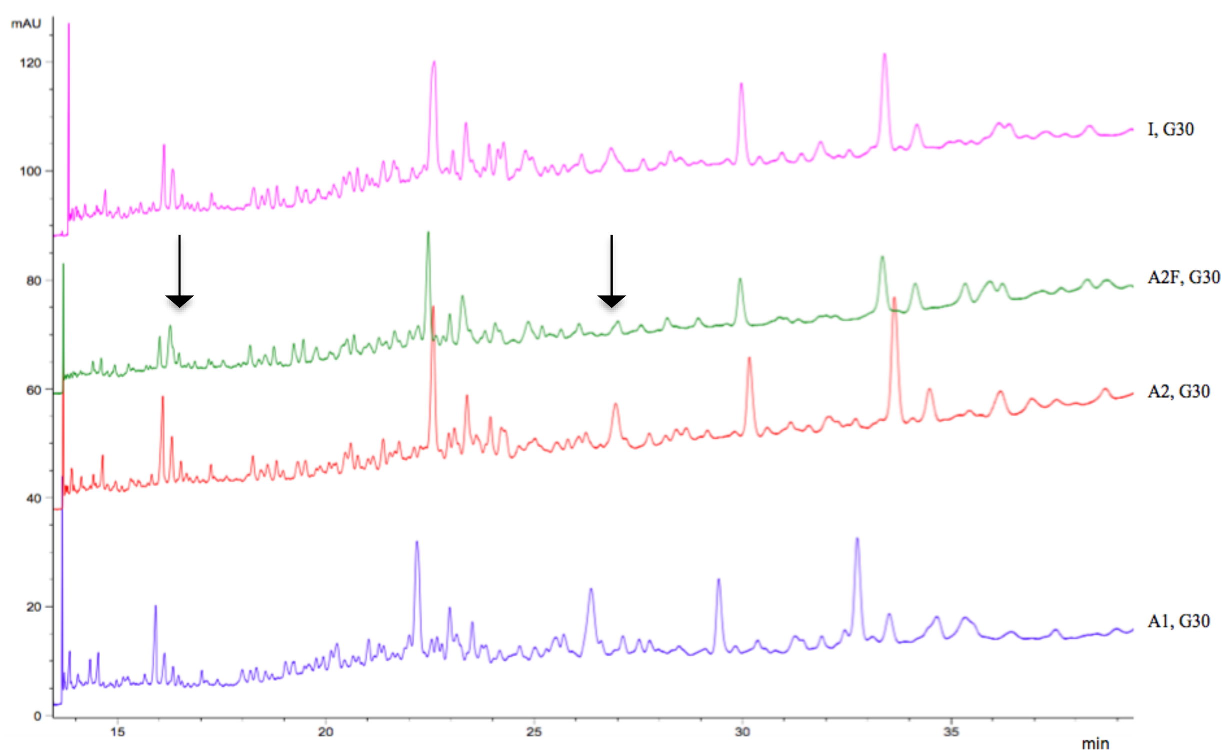

Figure 15. Electropherograms of milk samples with β-casein variant A1, A2, A2F and I, digested 30 min in the gastric phase. Arrows indicate visible differences in the degradation pattern.

Furthermore, the area under the graph was calculated for each digested sample as a reduction of total area in the undigested samples (100 %), presented in Table 8. The mean reduction of total area in the electropherogram was 27 % after 60 min gastric digestion. The lowest value was found for the I variant with an average total area of 62 % after digestion. The highest total area after gastric digestion was found in the A1 and A2 variant with 80 % of the area in the undigested samples. There were no major differences between gastric samples digested 30 and 60 min.

Table 8. Reduction of total area in the electropherogram of milk samples with different genetic variants (A1, A2, A2F and I) after 60 min of gastric digestion, compared with total area of undigested milk samples.

Genetic variant	A1	A2	A2F	I	Average
Reduction in total area (%)	20	20	28	38	27

Areas of peaks with known identity in undigested and digested samples were calculated by integration of peak area to estimate their resistant to enzymatic degradation during digestion. There were no visible differences in the degradation of proteins of different genetic variants. Figure 16 illustrates the degradation of individual proteins in milk samples by the average area of identified peaks. The calculated area revealed that the whey protein β-LG is mostly resistant in the gastric phase of the digestion, and the CNs are of most part degraded in this phase.

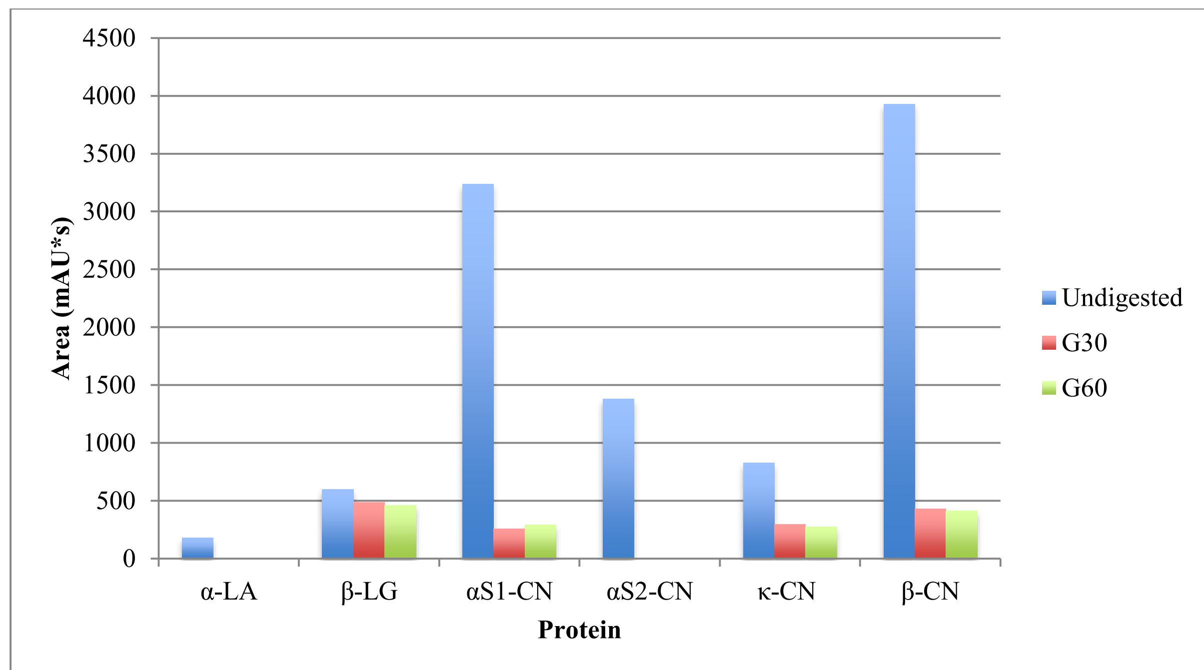

Figure 16. Average area of identified peaks in the electropherogram of undigested milk proteins and milk proteins after 30 (G30) and 60 (G60) min of gastric digestion.

3.3.5 Peptide Identification and Fragmentation

Peptides formed by β-CN during digestion were characterized by liquid chromatography coupled on-line to tandem mass spectrometry (LC-ESI-MS/MS). The spectra were recorded over the *m/z* range 50 to 3000 and submitted to Mascot database search using a bovine subset of the protein database, with known genetic variants of β-CN. Figure 17 illustrates the chromatogram of β-CN after gastric and duodenal digestion. The peaks illustrate all peptides and it was observed a higher amount of peptides in the duodenal phase compared to the gastric phase. All peptides identified with significant hits ($p < 0.05$) from the Mascot database search, or manually analysed spectra, are illustrated in Figure 18 and Figure 19, with

their respective position in the β-CN amino acid sequence. Digestion was carried out on both purified β-CN and skimmed milk. Digestion of purified β-CN gave a higher total amount of identified peptides than digestion of skimmed milk. An average of 26 (G60), 32 (D5), and 15 (D120) peptides were identified from purified β-CN, compared to 10 (G60), 25 (D5) and 14 (D120) peptides identified from β-CN as part of skimmed milk. In total, 114 different peptides were identified with 75 % sequence coverage.

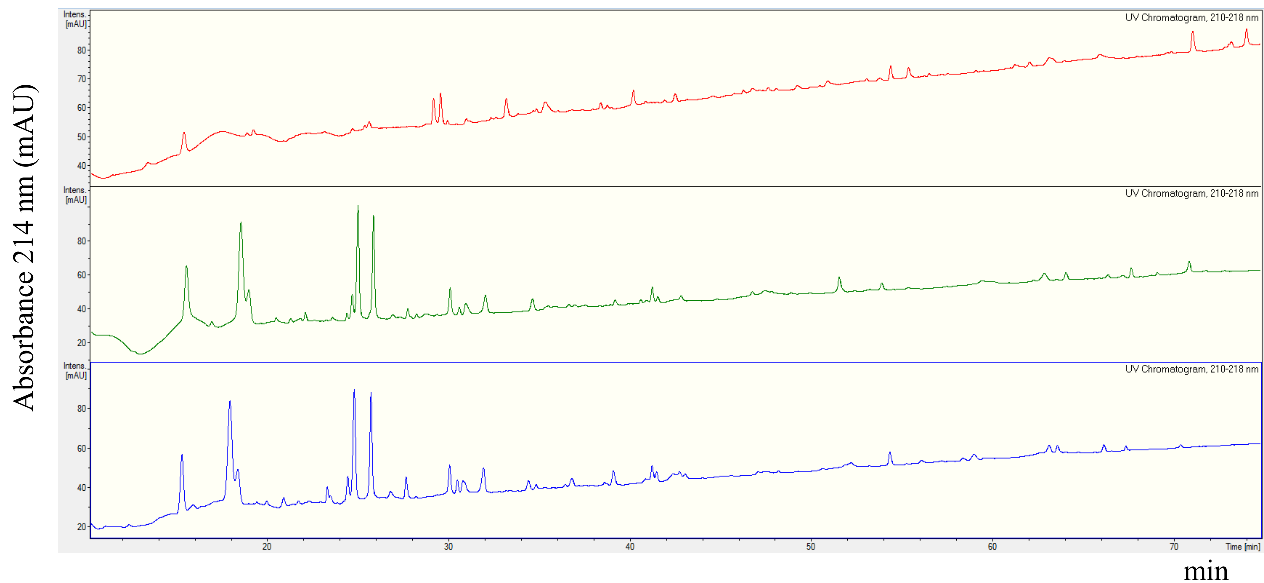

Figure 17. RP-HPLC chromatogram of digested β-casein. The peaks represent peptides separated by hydrophobicity. Red graph, 60 min of gastric digestion; green graph, 60 min of gastric digestion and 5 min of duodenal digestion; blue graph, 60 min of gastric digestion and 120 min of duodenal digestion.

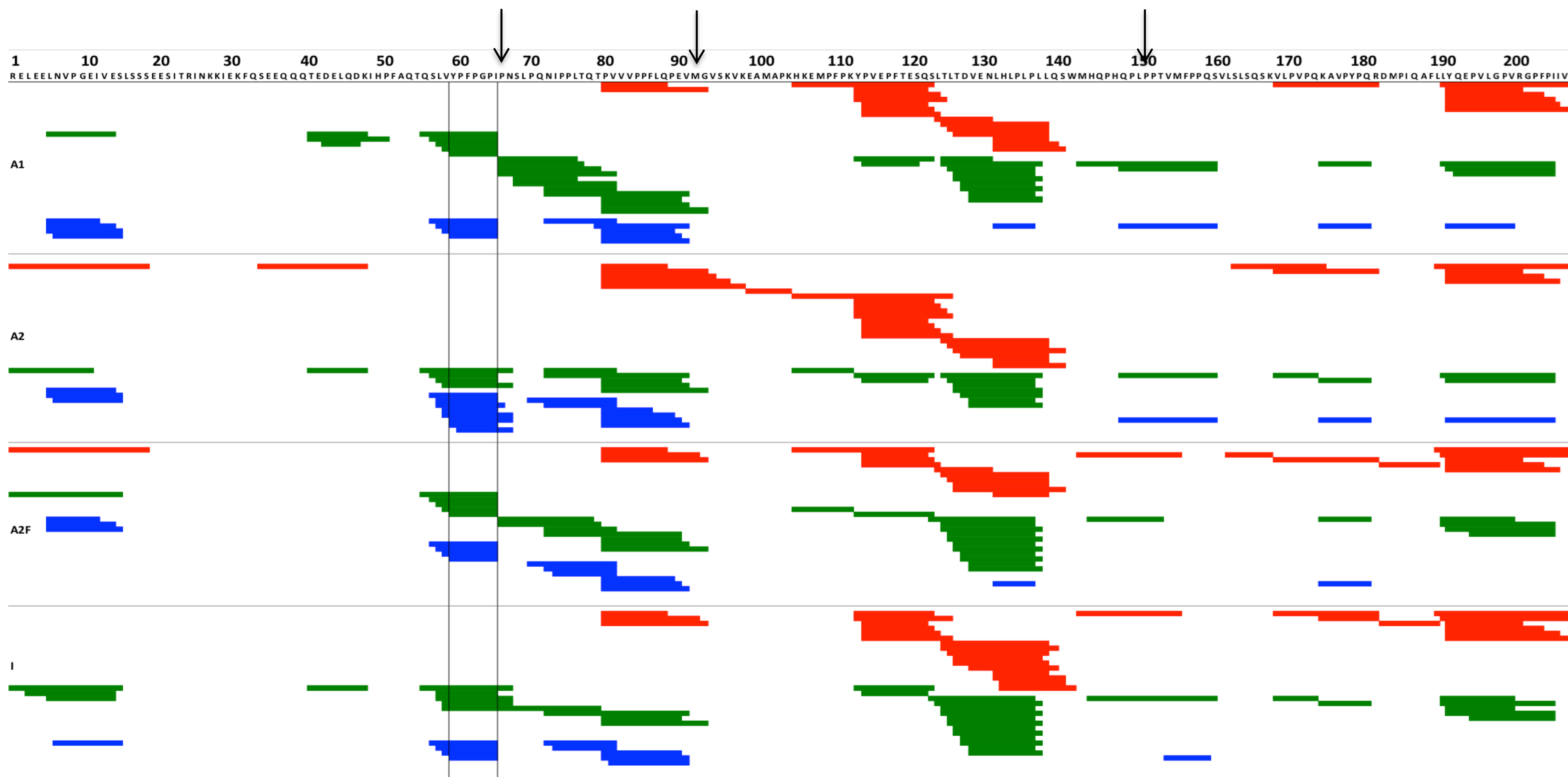

Figure 18. Peptide fractions identified by tandem mass spectrometry after *ex vivo* digestion of **purified β-casein** with genetic variants A1, A2, A2F and I. Peptides identified after 60 min of gastric digestion with HGJ are illustrated in red; peptides after 60 min of gastric digestion and 5 min of 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, F) P→H; position 93 (I) M→L; position 152 (F) P→L.

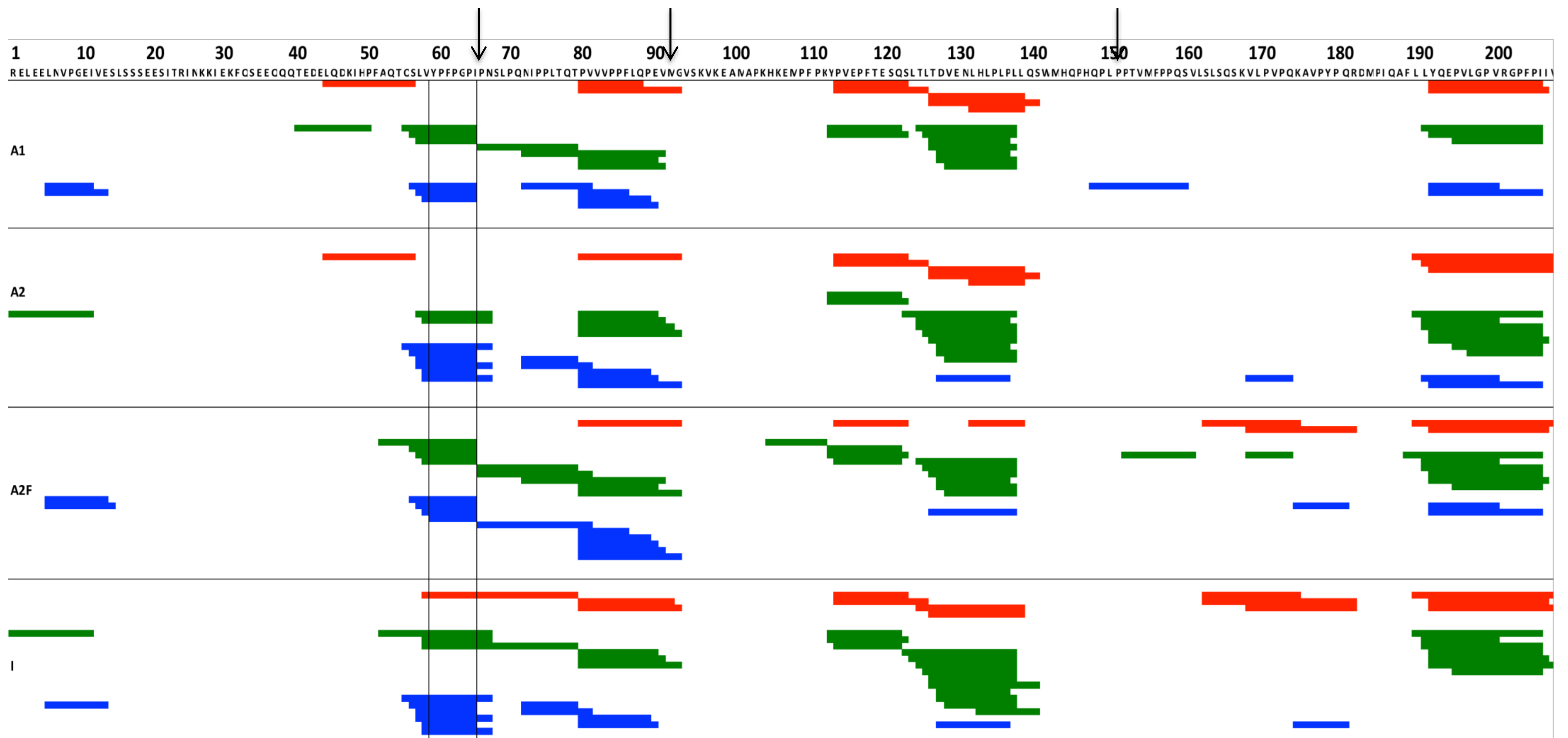

Figure 19. Peptide fractions identified by tandem mass spectrometry after *ex vivo* digestion of **skimmed milk** with β-casein genetic variants A1, A2, A2F and I. Peptides identified after 60 min of gastric digestion with HGJ are illustrated in red; peptides after 60 min of gastric digestion and 5 min 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, F) P→H; position 93 (I) M→L; position 152 (F) P→L.

All peptides identified within the position of polymorphism (position 67, 93 and 152) are presented in Table 9 and Table 10. After gastric digestion ten different peptides were identified within the area of amino acid substitution, where four peptides were identified from skimmed milk and nine from purified β-CN. These peptides were cleaved by the active pepsin in the HGJ. The peptide f(81-93), with P93L substitution, was exclusively found for the variant I after digestion of skimmed milk, however, the same peptide was found after gastric digestion of the heterozygous A2F purified β-CN. The remaining peptides identified were fractions cleaved at peptide bonds containing T, V and F.

After initial five minutes of duodenal digestion, the amount of peptides identified within the amino acid substitution region increased for all variants. A total of fourteen new peptides were identified, with three peptides being exclusive to the I variant. The most frequent peptides were found in the region of f(56-82). This region contains the P67H substitution for the A1 and F variant. Peptides cleaved N-term of position 67 was only identified from variant A1 and A2F, with three being exclusive to A1, and peptides cleaved C-term of position 67 was identified from all variants except A1. Furthermore, four peptides in the region of f(144-161) were identified from all variants. One peptide identified, which was exclusive for skimmed milk variant A2, was f(81-93). After full duodenal digestion for 120 min with HDJ, three new peptides were identified in the region of amino acid substitution. These peptides were exclusively identified from variant A2 after digestion of purified β-CN. The peptides were f(58-67), f(60-68) and f(61-68) cleaved at peptide bonds containing L, V, Y, and P. Other sites of cleavage throughout the duodenal phase of digestion occurred at peptide bonds containing H, Q and S.

The only peptide identified in all phases of the digestion within the region of amino acid substitution was f(81-94). This was identified for all variants, however, with P93L substitution for the I variant. The average length of all identified peptides decreased from thirteen amino acids in the gastric digestion to an average of nine amino acids at the end of duodenal digestion.

Table 9. Peptide fragments from regions with amino acid substitution identified by tandem mass spectrometry after *ex vivo* digestion of **purified β-CN**.

Digestion phase	Digested variant	Position[a]	Sequence[b]
Gastric 60 min	A2F,	81 - 93	(T)PVVVPPFLQPEV**M**(G)
	I	81 - 93	(T)PVVVPPFLQPEV**L**(G)
	A1, A2, A2F	81 - 94	(T)PVVVPPFLQPEV**M**G(V)
	I	81 - 94	(T)PVVVPPFLQPEV**L**G(V)
	A2	81 - 95	(T)PVVVPPFLQPEV**M**GV(S)
	A2	81 - 97	(T)PVVVPPFLQPEV**M**GVSK(V)
	A2	81 - 99	(T)PVVVPPFLQPEV**M**GVSKVK(E)
	A2F	144 - 156	(W)MHQPHQPL**L**PTVM(F)
	I	144 - 156	(W)MHQPHQPL**P**PTVM(F)
Duodenal 5 min	A2, I	56 - 68	(T)QSLVYPFPGPI**P**N(S)
	A2F	58 - 68	(S)LVYPFPGPI**P**N(S)
	A2, I	59 - 68	(L)VYPFPGPI**P**N(S)
	I	59 - 80	(L)VYPFPGPI**P**NSLPQNIPPLTQT(P)
	A1	67 - 77	(I)**H**NSLPQNIPPL(T)
	A1,	67 - 78	(I)**H**NSLPQNIPPLT(Q)
	A2F	67 - 79	(I)**H**NSLPQNIPPLTQ(T)
	A1, A2F	67 - 80	(I)**H**NSLPQNIPPLTQT(P)
	A1,	67 - 82	(I)**H**NSLPQNIPPLTQTPV(V)
	A1, A2, A2F	81 - 94	(T)PVVVPPFLQPEV**M**G(V)
	I	81 - 94	(T)PVVVPPFLQPEV**L**G(V)
	A1	144 - 161	(W)MHQPHQPL**P**PTVMFPPQS(V)
	A2F	145 - 154	(M)HQPHQPL**L**PT(V)
	I	145 - 161	(M)HQPHQPL**P**PTVMFPPQS(V)
	A1, A2	149 - 161	(H)QPL**P**PTVMFPPQS(V)
Duodenal 120 min	A2	58 - 67	(S)LVYPFPGPI**P**(N)
	A2	59 - 68	(L)VYPFPGPI**P**N(S)
	A2	60 - 68	(V)YPFPGPI**P**N(S)
	A2	61 - 68	(Y)PFPGPI**P**N(S)
	A1, A2	149 - 161	(H)QPL**P**PTVMFPPQS(V)

[a] Position in the mature β-CN sequence.

[b] Amino acid sequence using one-letter abbreviations and neighbouring amino acid in parentheses. Bold letters denote site of amino acid substitution.

Table 10. Peptide fragments from regions with amino acid substitution identified by tandem mass spectrometry after *ex vivo* digestion of **skimmed milk**.

Digestion phase	**Digested variant**	**Position[a]**	**Sequence[b]**
Gastric 60 min	I	59 - 80	(L)VYPFPGPI**P**NSLPQNIPPLTQT(P)
	I	81 - 93	(T)PVVVPPFLQPEV**L**(G)
	I	81 - 94	(T)PVVVPPFLQPEV**L**G(V)
	A1, A2, A2F	81 - 94	(T)PVVVPPFLQPEV**M**G(V)
Duodenal 5 min	I	53 - 68	(F)AQTQSLVYPFPGPI**P**N(S)
	A2	58 - 68	(S)LVYPFPGPI**P**N(S)
	A2, I	59 - 68	(L)VYPFPGPI**P**N(S)
	I	59 - 80	(L)VYPFPGPI**P**NSLPQNIPPLTQT(P)
	A2F	67 - 80	(I)**H**NSLPQNIPPLTQT)P)
	A1, A2F	67 - 82	(I)**H**NSLPQNIPPLTQTPV(V)
	A2	81 - 93	(T)PVVVPPFLQPEV**M**(G)
	A1, A2, A2F	81 - 94	(T)PVVVPPFLQPEV**M**G(V)
	I	81 - 94	(T)PVVVPPFLQPEV**L**G(V)
Duodenal 120 min	A2, I	56 - 68	(T)QSLVYPFPGPI**P**N(S)
	A2, I	58 - 68	(S)LVYPFPGPI**P**N(S)
	A2, I	59 - 68	(L)VYPFPGPI**P**N(S)
	A2	60 - 68	(V)YPFPGPI**P**N(S)
	A2F	67 - 82	(I)**H**NSLPQNIPPLTQTPV(V)
	A2, A2F	81 - 94	(T)PVVVPPFLQPEV**M**G(V)
	A1	149 - 161	(H)QPL**P**PTVMFPPQS(V)

[a] Position in the mature β-CN sequence.

[b] Amino acid sequence using one-letter abbreviations and neighbouring amino acid in parentheses. Bold letters denote site of amino acid substitution.

Gastrointestinal (GI) digestion of all β-CN variants resulted in identification of peptides previously described as bioactive, and is presented in Table 11. The ACE-inhibitory peptide f(6-14) was identified from all variants, as well as the opioid peptide f(60-66) (BCM7) after duodenal digestion. Furthermore, the ACE-inhibitory and antioxidative peptide f(59-68) was observed after duodenal digestion of β-CN A2 and I. Another ACE-inhibitory peptide, identified exclusively from the A2 variant, was f(60-68). In the gastric phase of digestion two bioactive peptides were found: the ACE-inhibitory peptide f(193-202) was identified from variants A1, A2 and A2F, and the antimicrobial and immunomodulatory peptide f(193-209) was identified from the variants A1, A2 and I.

Table 11. Peptide fragments with bioactivity (from literature) identified by tandem mass spectrometry after ex vivo digestion of skimmed milk and purified β-CN.

Phase of digestion	Digested variant [a]	Position [b]	Sequence [c]	Bioactivity	Reference
Duodenal	A1, A2, FA2, I	6 - 14	LNVPGEIVE	ACE-inhibitor	Gobbetti et al. (2002)
Duodenal	A2, I	59 - 68	VYPFPGPIPN	ACE-inhibitor, antioxidative	Eisele et al. (2013)
Duodenal	A1, A2, FA2, I	60 - 66	YPFPGPI	Opioid	Brantl et al. (1981)
Duodenal	A2	60 - 68	YPFPGPIPN	ACE-inhibitor	Saito et al. (2000)
Duodenal	A1, FA2	133 - 138	LHLPLP	ACE-inhibitor	Quirós et al. (2007)
Gastric	A1, A2, FA2	193 - 202	YQEPVLGPVR	ACE-inhibitor	Silva and Malcata (2005)
Gastric	A1, A2, I	193 - 209	YQEPVLGPVRGPFPIIV	Antimicrobial, immunomodulatory	Sandré et al. (2001)

[a] β-CN variant.
[b] Position in the mature β-CN sequence.
[c] Amino acid sequence using one-letter abbreviations.

Peptides that showed homology for a known amino acid sequence but was lacking the identity where manually analysed in BioTools and DataAnalysis. The software recorded individual masses, and the mass of interest was then evaluated by the identification of b and y ions in MASCOT. Figure 20 is an example of peptide identified by the fragmentation with b and y ions. The recognition was based on the LC-MS/MS of the protonated $[M+2H]^{2+}$ precursor ion with *m/z* 395.8, which corresponds to the monoprotonated mass of BCM7. The peptide illustrated is f(60-66) identified from variant A1 after 5 min of duodenal digestion. In addition to the identification of BCM7, several BCM7-like peptides were identified after digestion of all variants of purified β-CN (presented in Table 12). These peptides were cleaved at the C-terminal end of position 66 and at the N-terminal end at position 56-59. The peptide f(59-66) is also referred to as V-BCM7, as it contains V instead of Y N-term.

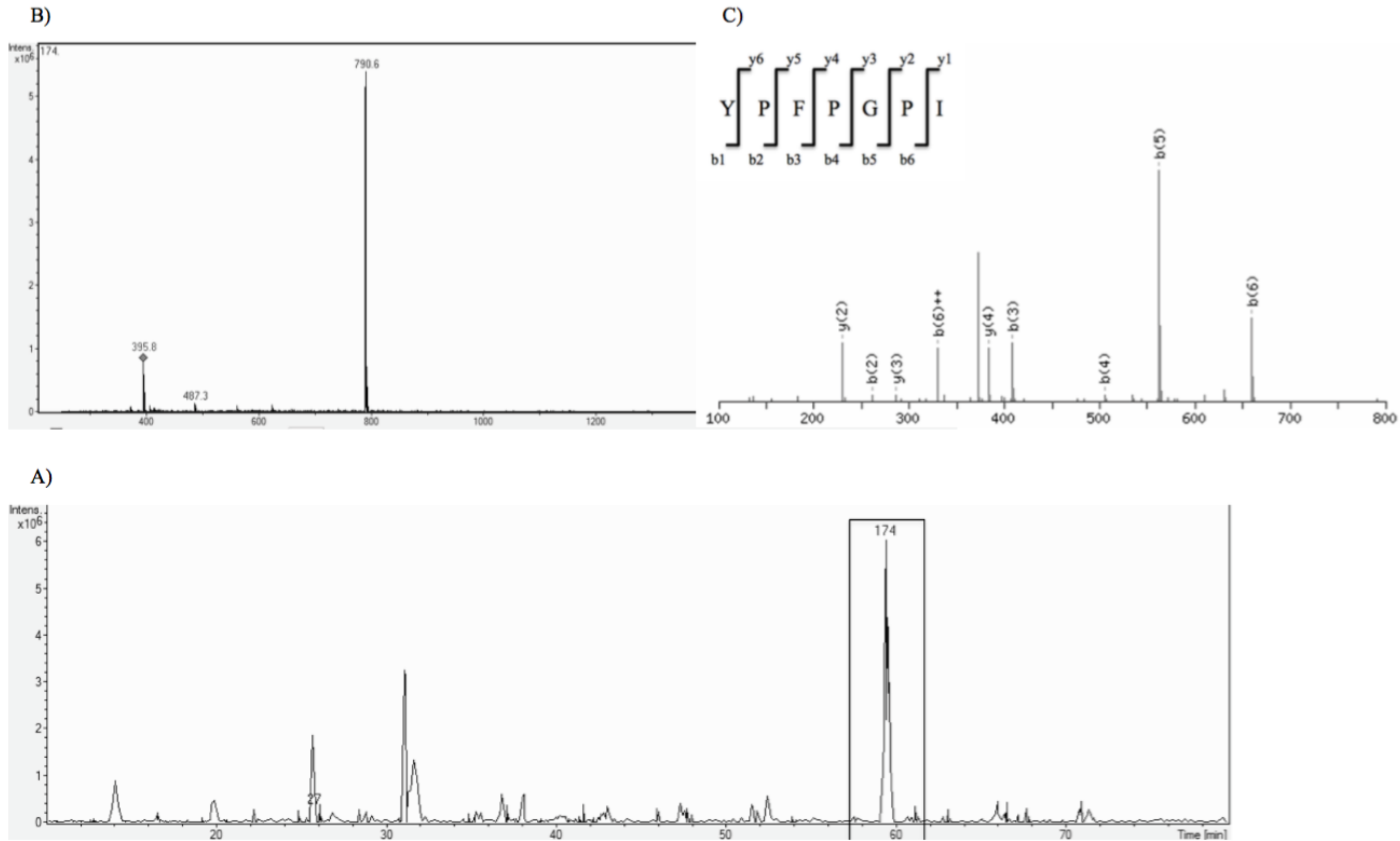

Figure 20. A) Base peak chromatogram for the mass 395.8 of purified β-CN after 60 min of gastric digestion with HGJ and 5 min of duodenal digestion with HDJ. B) Mass spectrum of marked peak in (A). C) Tandem mass spectrum of ion with *m/z* 790.6 from (B). Following sequence identification and MASCOT search, the MS/MS spectrum matches the β-CN sequence f(60-66) (BCM7).

Table 12. Peptide fragments cleaved N-term of P67 identified by LC-ESI-MS/MS after *ex vivo* digestion of purified β-CN.

Phase of digestion	**Digested variant**	**Position**[a]	**Sequence** [b]
Gastric 60 min	No sequence found		
Duodenal 5 min	A1, FA2	56 - 66	QSLVYPFPGPI
	A1, A2, FA2	57 - 66	SLVYPFPGPI
	A1, A2, FA2, I	58 - 66	LVYPFPGPI
	A1, FA2	59 - 66	VYPFPGPI
Duodenal 120 min	A1, A2, FA2, I	57 - 66	SLVYPFPGPI
	A1, A2, FA2, I	58 - 66	LVYPFPGPI
	A1, A2, FA2, I	59 - 66	VYPFPGPI

[a] Position in the mature β-CN sequence.

[b] Amino acid sequence using one-letter abbreviations.

4 Discussion

A number of *in vitro* and *in vivo* studies have described the effect of A1 β-CN ingestion and the release of β-casomorphin-7 (De Noni 2008; Jinsmaa & Yoshikawa 1999; Tailford et al. 2003). In a review on the potential health-effects of BCM7, EFSA (2009) concluded that a formal risk assessment of the peptide is not needed, and that more comprehensive studies are desired to eliminate other factors that can be responsible for the outcomes in previous studies. The present study was carried out to establish the degradation profile of β-casein genetic variant A1, A2, A2F and I in bovine milk after *ex vivo* gastrointestinal digestion. The main focus was identification of bioactive peptides.

β-Casein was isolated from skimmed milk to evaluate the digestion of pure β-CN compared to skimmed milk. The average purity of the isolated β-CN was similar to results previously reported by Petrat-Melin et al. (2015), which used the same method of purification. This method for determination of protein purity 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 (Fox & McSweeney 1998; Petrat-Melin 2014). However, the yield of β-CN was in average 27% of total β-CN in skimmed milk. The main reason for this is that the β-CN loosely bound to other CNs through hydrophobic interactions were isolated, and the larger amount of β-CN that forms nanoclusters with serine groups did not precipitate after cold storage and ultracentrifugation (Petrat-Melin 2014). These CNs were still bound in the micelle structure. Surprisingly, the A2F β-CN did not present itself as heterozygote in the LC-MS analysis, or in the electropherogram by CE. However, this variant was identified by DNA sequencing of the gene at Aarhus University, and the heterozygote variant A2F was identified. Peptide identification by LC-ESI-MS/MS showed amino acids at polymorphic site representing both variant F and A2.

Skimmed milk and purified β-CN were digested according to the INFOGEST protocol (Minekus et al. 2014); however, some modifications were necessary due to the use of human enzymes. The human digestive juices comprise various isoforms, and this may broaden their

spectrum of cleavage. Subsequently, the enzyme activities in the human juices were calculated on the basis of the ratio between substrate and enzyme secretion (vol:vol) in an “in house” protocol (Ulleberg et al. 2011). A pilot study of the digested proteins on SDS-PAGE was done to ensure that the enzyme activity was sufficient for hydrolysis of proteins and compared to earlier data from Islam et al. (2015a). The degradation profiles also proved that the time chosen in the gastric and duodenal phases were sufficient for enzymatic digestion of milk proteins. 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. (2015a), CNs showed almost complete degradation after 40 min in the gastric phase with HGJ, and the remaining proteins were degraded after 5 min in the duodenal phase with HDJ. These results laid the basis for the chosen digestion time for the present study. Consequently, all CNs were digested after 60 min in the gastric phase while the whey proteins α-LA and β-LG were resistant. This is partly in consistency with a previous study on the digestion of milk from different species by human gastrointestinal enzymes (Inglingstad et al. 2010). Inglingstad et al. (2010) observed complete resistance of whey proteins after gastric digestion, however, only 30 % of CNs were digested in the gastric phase, and completely digested in the duodenal phase. Islam et al. (2015b) also observed resistance of degradation of whey proteins in the gastric digestion. The resistance during gastric digestion of whey proteins may be explained by their globular structure and ability to form disulfide bonds. Both α-LA and β-LG contains cysteine residues in their amino acid sequence, which maintains the structure of the protein and buries sites of hydrophobic and aromatic amino acid residues which are more susceptible to pepsin cleavage (Reddy et al. 1988). Furthermore, the denser bands observed in the region of β-LG after 60 min of gastric digestion may be accumulation of peptides formed with the same molecular weight from other proteins, thus an illusion of whey proteins’ resistance to gastric digestion. There were no visible differences in the degradation pattern of the genetic variants. It is expected that the genetic variants will produce different peptides, however, these peptides may be of low concentration and smaller than 10 kDa, and is therefore not visible in the gel. Petrat-Melin et al. (2015) observed differences in the degradation profile of β-CN after digestion with pancreatic enzymes, where a peptide band was absent around 4 kDa from digestion of β-CN

variant B. This variant has the amino acid substitution S122R, where R is more prone to trypsin cleavage, thus possibly creating new cleavage sites. Furthermore, in the present study, some faint bands around 10-20 kDa were visible after 60 min of gastric digestion with purified β-CN. 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. The analysis performed in this study by SDS-PAGE served to illustrate the digestion pattern. Consequently, neither quantification nor identification of peptides generated during digestion were performed using this method.

The degree of hydrolysis (DH) showed clear differences between gastric and duodenal phase of digestion, however the overall DH of purified β-CN was considerably higher than of skimmed milk. This could be explained by the enzyme activity during digestion of the proteins. Both skimmed milk and purified β-CN were digested with the enzyme activity needed for 1 mL liquid food, according to the INFOGEST protocol (Minekus et al. 2014); however, the amount of substrate (skimmed milk or purified β-CN) was determined by equal amount of β-CN in the sample. Consequently, the skimmed milk contained a higher total protein content (33 mg/mL), as it also contained other CNs and whey proteins. During the digestion, the amount of peptide bonds hydrolysed in the skimmed milk samples may therefore be lower compared to total peptide bonds present. After measuring the DH of the hydrolysates, the values were much higher than expected. Therefore, the DH of the HGJ and HDJ were also measured, and used as blank samples to adjust the DH value of the hydrolysates. The DH measured after gastric phase was higher than found in a previous study by Petrat-Melin et al. (2015). The most plausible explanation for the high DH observed in the present study may be the substrate-enzyme ratio applied, and the possible broad spectrum of cleavage by the human juices. As expected, there were no significant ($p < 0.05$) differences in DH between the genetic variants of β-CN. This is expected because the DH is calculated by the molar extinction coefficient, which is determined by the amino acid residues Y, W, and C. All four variants digested in this study have the same amount of these residues, and therefore, they are not expected to differ in DH value. Comparing the high digestibility of β-CN observed from the gel electrophoresis, with the rather low DH after gastric digestion, it is plausible to suggest that several larger peptide fragments have been produced. This could be explained by the structure of β-CN, where hydrophobic stretches within the protein may

aggregate and delay the hydrolysis by gastric juices (Ossowski et al. 2012). Consequently, peptic cleavage of β-CN occurs, although large stretches of the protein stay intact.

The peptide profile identified by tandem mass spectrometry was not performed for undigested milk; thus, it is dubious to suggest that the peptides observed after digestion could already be present in the milk. However, CE analysis was performed on the undigested milk and purified β-CN, and the electropherograms showed no degradation in the undigested milk. Furthermore, the reduction of total area in the graph of the digested samples indicated that the protein had been degraded, and the resulting amino acids and small peptides generated may not have been detected by absorbance at 214 nm. A reduction of the individual CNs after gastric digestion was also observed in the electropherograms, however, as seen in the degradation profile by SDS-PAGE, β-LG was mostly resistant, and showed little degradation in the electropherograms. Capillary electrophoresis was only performed on gastric samples; thus, peptides formed in the duodenal phase of digestion were not observed by this method.

Furthermore, the integrated peak area of the individual CN could indicate the purity of the β-CN samples. Compared with LC-MS measurements and absorption at 280 nm, the CE obtained lower values. However, the calculated purity from LC-MS and spectrophotometric measurements was based on the molecular extinction coefficient, of the individual proteins, and the absolute protein content could therefore be estimated with higher accuracy.

The sequence coverage by mass spectrometry after digestion of purified β-CN was about 75 %, which is in consistence with a previous study reported by Schmelzer et al. (2004), who identified 41 peptides with 75 5 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 mass spectrometry approaches, based on both Matrix Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-TOF) and LC-ESI-MS/MS. In the present study a total of 114 peptides were identified from purified β-CN after *ex vivo* digestion. The use of purified β-CN seems more effective in approaches for digestion, compared to skimmed milk. Most likely due to a purer sample of β-CN, and by avoiding disturbance of other proteins when identification methods are applied. The sequence

coverage and peptides observed were similar to the abovementioned, though the present study was based on both gastric and duodenal digestion.

In the gastric phase of digestion, the active enzyme pepsin hydrolyses the proteins. This enzyme cleaves preferably at sites of F, L and Y, however, pepsin can cleave with a more complex action as well, depending on the amino acid combination upstream or downstream of the cleavage site (Tang 1963). Several sites of cleavage were observed after digestion, including at the site of residue P. Proline is considered rather resistant to pepsin cleavage, thus the use of human juices may have enhanced the diverse cleavage, as it is not pure commercial pepsin, but a complex mixture of different isoforms of gastric enzymes (Devle et al. 2014). After digestion of skimmed milk variant I, the peptide f(81-93) (PVVVPPFLQPEVL) was identified. This variant contains L in position 93, and is therefore more prone to cleavage by pepsin. Surprisingly, the same peptide was identified from digestion of purified β-CN variant A2F, containing M in position 93. The V residue in the penultimate position can, in fact, affect the action of pepsin and also trigger cleavage at position 93 (MEROPS 2016). The *in silico* digestion did not produce any of the fragments identified in the gastric phase by HGJ, however it did produce cleavage sites at residues L and Y, as is expected from hydrolysis by pepsin. Furthermore, all samples were filtered through a 10 kDa cut-off filter to remove larger substances that could interfere or not be detected in the MS. Consequently, fewer but longer peptides were identified in the gastric phase. The gastric samples did most likely contain some larger peptides or undigested proteins that were removed and therefore not identified.

After digestion with human duodenal juice (HDJ), cleavage sites at R and K residues were observed, in addition to those observed in the gastric phase. The most frequent peptides exclusively observed after duodenal digestion were peptides derived from the region of f(56-82) in the mature β-CN, 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. Peptides cleaved N-terminal of position 67 were only identified from variant A1 and A2F, which contains the amino acid substitution P67H in variant A1 and F. However, several peptides in the region of f(56-66) were identified among all variants. This indicates that the hydrolysis by human duodenal juices is not only dependent on the residue H in position 67. Furthermore, peptides in the region of f(67-82) in variant A2 and I were missing in the

peptide profile, but fractions downstream of this cleavage site were identified. This could be a cause of non-identified peptides, rather than non-existing peptides.

Another cleavage site observed exclusively in the duodenal phase were at residues R and K, which indicates the action of hydrolysis by trypsin. Furthermore, *in silico* digestion of variant A1 and F generated the peptide f(61-67) with C-terminal cleavage at residue H in position 67 by chymotrypsin. Cleavage sites at residues L and P were also produced by algorithms in *in silico* digestion, corresponding to preferable cleavage sites of chymotrypsin. Moreover, three peptides from duodenal digestion were identified from variant A2, A2F and I in the region f(58-68), containing P in position 67, which suggests that H is more prone to cleavage by chymotrypsin. The peptide f(60-68) (YPFPGPIPN) was identified exclusively after 120 min duodenal digestion of variant A2. This is a bioactive peptide (BAP) reported by Saito et al. (2000), where the residues I and P in the C-terminal end of the peptide showed potent ACE-inhibitory effect. However, the author reported that this peptide had rather low antihypertensivity, which is explained by the large size of the molecule that requires further digestion by intestinal enzymes before absorption. Furthermore, one similar ACE-inhibiting peptide, f(59-68), also possessing antioxidative activity, was identified from variant A2 and I. This peptide is referred to as V-BCM-9, and showed to increase the IC_{50} value almost 22-fold, caused by the addition of V in the N-terminal end of the BCM peptide (Eisele et al. 2013). Another study performed by Petrat-Melin et al. (2015) found that the A1 variant of this same peptide, with the residue H in position 67, had a 3-fold decrease in IC_{50} value, making the peptide a stronger ACE inhibitory agent than the A2 variant of V-BCM-9. In addition to the aforementioned BAPs, two other peptides with ACE-inhibitory properties were identified: the peptide f(6-14), previously identified by Gobbetti et al. (2000), and the peptide f(133-138), identified from variant A1 and A2F after duodenal digestion, previously identified by Quirós et al. (2007). These peptide sequences have been found after fermentation of milk with lactic acid bacteria.

The opioid peptide β-casomorphin-7 was identified after digestion of all genetic variants as it appeared after 120 min of duodenal digestion of variant A2 and I, compared to variant A1 and A2F, where it was identified after initial 5 min duodenal digestion. As explained above, the H residue in position 67 is considered more prone to cleavage by proteolytic enzymes; hence, the cleavage occurs at a higher rate in the duodenal digestion when this residue is

present. However, the action of the duodenal juices increases with time, making sites of cleavage more diverse, and the residues Q, S, P and N were also observed as potent cleavage sites, as previously reported by Petrat-Melin (2014). This suggests that the residue P in position 67 of variant A2 and I may affect the proteolytic cleavage, as seen in the gastric phase, even though it is known to be relatively resistant to proteolytic cleavage.

Furthermore, four peptides in the region of f(56-66) were identified after duodenal digestion of all variants. These peptides are referred to as BCM-like peptides, and the identification of these supports the theory of generation of BCM7 after digestion of both variant A1 and A2 β-CN. However, the N-terminal in BCM-like peptides does not contain the same amino acid as BCM7. This will affect the peptides ability of absorption, transport and of binding 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 et al. 2007; De Noni 2008; Hernández-Ledesma et al. 2004; Schmelzer et al. 2004; Ul Haq et al. 2015). In a study performed on different genetic variants of β-CN, generation of BCM7 was observed from variant A1 and B (which also contains H in position 67) after simulated GI digestion (De Noni 2008). The author did not observe release of BCM7 from variant A2. Furthermore, in a study performed by Cieslinska et al. (2007) hydrolysis of A2 β-CN showed to release BCM7, however, the concentration of the peptide was four times higher when produced from the homozygous A1 β-CN. There is no other data from literature that reveal the generation of BCM7 from A2 β-CN during simulated GI digestion with commercial enzymes; moreover, the study by Cieslinska and co-workers was not performed under true conditions as the peptic digestion lasted for 24 h. In addition, the peptide profile was performed by HPLC/UV, which is not as reliable as mass spectrometry based methods (De Noni 2008).

As indicated above, there is a diverse generation of bioactive peptides deriving from CN during GI digestion. In addition to the abovementioned, 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 the present study did not allow identification of peptides smaller than five amino acids long. Other methods need to be applied to identify bioactive peptides such as the ACE-inhibitors VPP and IPP. Di- and tripeptides are often isobaric and co-eluting, which makes them difficult to separate and

identify (Lahrichi et al. 2013). In addition, the degree of hydrolysis is not in consistency with data contained from the peptide profile. The average length of peptide in late duodenal phase was calculated to 1.3 amino acids, although the peptide profile showed length ranging from 5 to 15 amino acids.

Even though β-casomorphin-7 was detected after digestion of β-CN with human gastrointestinal enzymes, it may not be absorbed intact in the intestines; and if it is absorbed, it may still be broken down by proteases (e.g. dipeptidyl peptidase-4) before reaching its final target (e.g. opioid receptors). Thus, there is not enough evidence based only on digestive experiment that BCM7 can be linked to diseases such as autism, cardiovascular diseases, diabetes, etc. Guerra et al. (2012) reviewed different approaches for simulating human digestion. Static and dynamic models were discussed concerning their limitations and challenges. The overall concluding remark was that it is impossible to fully mimic the digestive parameters *in vivo* in a single simulated digestion model (*in vitro/ex vivo*). Digestive processes such as hormonal and nervous control, feedback mechanisms, mucosal cell activity, peristaltic movements and the influence of the immune system are complex parameters to fit into one model. Subsequently, combinatorial approaches have been performed for the evaluation of intestinal permeability and the bioavailability of digested compounds (Deat et al. 2009; Foltz et al. 2008; Osborne et al. 2014; Petrat-Melin 2014). The properties of epithelial cells in the small intestines have been studied for designing a model that exerts the mechanisms of absorption. Caco-2 monolayers are widely used as a potent *in vitro* model to predict absorption of bioactive peptides. Foltz et al. (2008) observed that ACE-inhibitory tri-peptides were absorbed partially undegraded, and the permeability of the peptide increased with increasing physiological relevance of absorption, such as *ex vivo*/*in situ* approaches. Furthermore, Quirós et al. (2008) reported that the ACE-inhibitory hexapeptide LHLPLP was partially hydrolysed to HLPLP by brush border enzymes, demonstrating that this pentapeptide was responsible for the antihypertensive effect of LHLPLP. Hydrolysis of BAPs by brush border enzymes has been reported by Iwan et al. (2008), that demonstrated the effect of dipeptidyl peptidase-4 (DPP4) on BCM7. The authors found that the presence of DPP4-inhibitor increased transport of BCM7, and that DPP4 was the main factor of limiting the half-life of opioid peptides. Osborne et al. (2014) reported similar results conducting peptide permeability studies with Caco-2 cell monolayer, where rapid hydrolysis of BCM7 was observed generating three peptide metabolites, YP, GPI and

FPGPI. Furthermore, individuals with leaky guts may be more prone to physiological effects of bioactive peptides 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 were no evidence linking autism to opioid peptides or to DPP4 deficiency (Cass et al. 2008; Hunter et al. 2003).

The European Food Safety Authority has published a review on the possible health effects of β-casomorphins and related bioactive peptides (EFSA 2009). All available scientific literature and studies (human, animal and *in vitro* trials) on the subject were evaluated, and the overall concluding remarks were that there is not enough evidence to support the link between consumption of casein and the incidence of non-communicable diseases caused by BCMs. One of the main concerns rejecting a risk assessment of BCM7 is the lack of *in vivo* studies detecting BCM7 molecules in blood after milk consumption. However, in a later study, Kost et al. (2009) was able to detect BCM7 in blood plasma of infants after consumption of bovine BCM7.

4.1 Conclusion and Future Prospect

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 amino acid substitutions can affect the hydrolysis by gastrointestinal proteases, thus effecting peptides formed. It has also been established in the present study that digestion of β-CNs leads to generation of several bioactive peptides; however, the release of BCM7 is potentially not dependent on the genetic variant with residue H at position 67 in the amino acid sequence of β-CN. Moreover, quantification of this peptide was not performed and further work should aim to quantify BCM7 in the digested variants of β-CN, to determine whether the amount is equal among the variants, especially between variant A1 and A2. In addition, there is a need for evaluating the bioaccessibility of generated BAPs, as their function after release in the intestines is rather ambiguous. Irrespective of the ongoing debate regarding health effects of BCM7 and other peptides, the data attained in this study brings additional clarification on the possible generation of BAPs and the importance of understanding the complexity of which parameters are regulating digestion, absorption, and transport of bioactive peptides.

With these aspects in mind, further work on quantification of peptides could be achieved by:

- Run synthesised BCM7 as a standard in capillary electrophoresis and comparing with peptides in all phases of digestion.
- Use extracted ion chromatogram of identified peptides to compare concentration between samples of different genetic variants.
- Apply quantitative proteomics analysis including triple quadrupole instrument using the multiple reaction monitoring technique.
- Amino acid analysis for a complete degradation profile on both peptides and free amino acids.
- Further identification of ACE-inhibitory di- and tripeptides.

In respect to the ongoing debate on health effects of BCM7 and other related peptides, more studies are needed to elucidate the diverse results in this field concerning:

- Absorption of BAPs through Caco-2 monolayers with respect to intestinal permeability and DPP4 activity.
- Assessment of elevated BCM7 level in blood plasma after bovine milk consumption.

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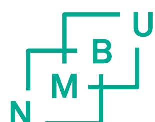
6 Appendix – Single-Letter Amino Acid Codes

A – Alanine
C – Cysteine
D – Aspartic acid
E – Glutamic acid
F – Phenylalanine
G – Glycine
H – Histidine
I – Isoleucine
K – Lysine
L – Leucine
M – Methionine
N – Asparagine
P – Proline
Q – Glutamine
R – Arginine
S – Serine
T – Threonine
V – Valine
W – Tryptophan
Y – Tyrosine



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