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This thesis is dedicated in memory of my father

TABLE OF CONTENTS

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Ås, October 2011 Ellen K. Ulleberg

ABBREVIATIONS

α-LA	Alpha-lactalbumin
α_{s1}-, α_{s2}-, β-, κ-CN	Alpha$_{s1}$-, alpha$_{s2}$-, beta-, kappa-casein
β-LG	Beta-lactoglobulin
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
Con A	Concanavalin A
CPP	Corolase PP
DC	Dendritic cell
GMP	Glycomacropeptide
HDJ	Human duodenal juice
HGJ	Human gastric juice
HPLC	High performance liquid chromatography
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LC-MS	Liquid chromatography – mass spectrometry
LF	Lactoferrin
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MS	Mass spectrometry
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PHA	Phytohemagglutinin
Q-TOF	Quadrupole - time of flight
SA	Serum albumin
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SK	Skimmed milk
Tc	T cytotoxic cell
TCR	T cell antigen receptor
Th	T helper cell
WPCC	Cow whey protein concentrate
WPCG	Goat whey protein concentrate

LIST OF PAPERS

Paper I

Ulleberg, EK, Comi, I, Holm, H, Herud, EB, Jacobsen, M & Vegarud, GE. Human gastrointestinal juices intended for use in *in vitro* digestion models. Food Digestion, In press.

Paper II

Eriksen, EK, Holm, H, Jensen, E, Aaboe, R, Devold, TG, Jacobsen, M & Vegarud, GE (2010). Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. British Journal of Nutrition, 104, 374-381.*

Paper III

Almaas, H, Eriksen, EK, Sekse, C, Comi, I, Flengsrud, R, Holm, H, Jensen, E, Jacobsen, M, Langsrud, T & Vegarud, GE (2011). Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juices. British Journal of Nutrition, 106, 896-905.*

Paper IV

Eriksen, EK, Vegarud, GE, Langsrud, T, Almaas, H & Lea, T (2008). Effect of milk proteins and their hydrolysates on *in vitro* immune responses. Small Ruminant Research, 79, 29-37. 5th IDF Symposium on the Challenge to Sheep and Goats Milk Sectors.*

Paper V

Ulleberg, EK, Vegarud, GE, Jensen, E, Holm, H & Lea, T. Immune cell cytokine profiles induced by different milk derived peptides (manuscript).

*The candidates last name was changed from Eriksen to Ulleberg in March 2011

SUMMARY

The objectives of this thesis were to characterise human gastrointestinal juices, to identify peptides produced after *in vitro* digestion of whey proteins by these juices, and to screen for immunomodulating properties of whey hydrolysates and single synthetic peptides.

The volumes of gastric and duodenal juices as well as their pH differed between individuals. Large individual variations in the enzyme activities and amounts of duodenal bile acids were also observed.

Frozen storage of gastric juice resulted in a rapid decrease in pepsin activity. The stability was not improved by adding 13% glycerol or by increasing the pH to 4. In duodenal juice the lipase activity on the other hand remained stable for 4 months and thereafter decreased. Total proteolytic and amylase activities remained stable.

A two-step *in vitro* digestion model was performed to simulate digestion of whey proteins in the stomach and the duodenum. Protein degradation patterns were shown by SDS-PAGE and generated peptides were identified by nano-LC-MS/MS. Digestion using human gastroduodenal juices was compared with digestion by porcine digestive enzymes at similar enzyme activities. In addition, gastric digestion was performed at pH 2, 4 or 6 to account for normal pH variations. The results showed that the lower the gastric pH, the more degraded were the proteins in both steps. Porcine enzymes, especially pepsin, were more efficient at degrading the whey proteins compared to the human juices. The most extensive digestion of the proteins was observed after gastric digestion at pH 2 with porcine enzymes.

The present study showed that digestion of whey proteins with human gastric and duodenal juices generated peptides of immunomodulatory nature. Twenty-two peptides identified within the digested caprine whey concentrate were synthesised. While whey proteins and hydrolysates demonstrated a dose-dependent inhibitory effect on T cell proliferation, the effect could not be explained by any of the single synthetic peptides alone. The whey protein samples were moreover shown not to be toxic to the cells and they did not induce apoptosis.

The synthetic peptides and fractions of digested whey protein increased the secretion of IL-1β and IL-12, while the secretion of IL-4 and IL-5 was reduced by many samples. The peptides only affected the DC secretion of IL-6 and IL-8, whereas the levels of IL-10, IL-12 and IFN-γ were unaltered. Interestingly, each peptide seemed to induce unique changes in the cytokine profiles and could therefore potentially display different immunomodulating effects *in vivo*.

SAMMENDRAG

Formålene med dette studiet var å karakterisere humane mage- og tarmsekreter, å identifisere peptider dannet ved *in vitro* fordøyelse av myseproteiner, og å lete etter immunmodulerende egenskaper ved myseproteinhydrolysater og syntetiske mysepeptider.

Sekreterte volum av mage-og tarmsaft og pH i disse varierte mellom individer. I tillegg ble det registrert store variasjoner i enzymaktiviter og i mengde gallesalter i de individuelle fordøyelsessekretene.

Ved fryselagring av sekretene avtok pepsinaktiviteten i magesaften raskt. Stabiliteten ble ikke bedret ved å tilsette 13% glycerol, eller ved å øke pH til 4. Lipaseaktiviteten i tarmsaften holdt seg stabil i 4 måneder, men avtok deretter. Total proteolytisk- og amylase-aktivitet holdt seg stabile under lagring.

En to-trinns *in vitro* fordøyelsesmodell ble utført for å simulere fordøyelse i mage og tarm. Fordøyelse ved human mage- og tarmsaft ble sammenlignet med fordøyelse ved kommersielle fordøyelsesenzymer fra gris. I tillegg, ble "magetrinnet" utført ved pH 2, 4 or 6 for å inkludere normale variasjoner i pH i magesekken. Resultatene viste at jo lavere pH var ved fordøyelse i magen, jo mer degraderete ble proteinene i begge trinn. Enzymene fra gris, og da særlig pepsin, var mer effektivt til å degradere myseproteinene sammenlignet med mage- og tarmsaften. Mest fordøyd ble proteinen etter fordøyelse ved pH 2 i magetrinnet og med kommersielle rensede enzymer fra gris.

Denne studien viste at fordøyelse av myseproteiner med mage- og tarmsaft førte til dannelse av immunomodulerende peptider. 22 peptider som var identifisert i det fordøyde myseprotein konsentratet ble syntetisert. Myseproteiner og hydrolysater viste en dose-avhengig inhibering på T-celle proliferasjon, men effekten kunne ikke forklares ved ett av de syntetiske peptidene alene. Myseproteinene viste ingen toksisk effekt på cellene og induserte ikke apoptose.

De syntetiske peptidene og fraksjoner av fordøyd myseprotein økte T cellenes sekresjon av of IL-1β og IL-12, mens sekresjonen av IL-4 og IL-5 avtok i nærvær av mange av disse prøvene. De syntetiske peptidene hadde kun effekt på den dendritiske cellenes sekresjon av IL-6 and IL-8, mens nivåene av IL-10, IL-12 og IFN-γ forble uendret. Særlig interesant var det at de enkelte peptidene så ut til å indusere unike endringer i cytokinprofilene til både T celler og dendritiske celler. Dette kan tyde på at de potensielt har ulike immunmodulating effekter *in vivo*.

1. INTRODUCTION

1.1 DIGESTION OF PROTEINS

Humans daily ingest proteins of varying amino acid composition, molecular weight and structure. Some of these amino acids are essential to humans since they cannot be produced in the body and have to be provided by what we eat. A number of mechanisms ensure that this diverse population of proteins is reduced to shorter peptides and amino acids to facilitate their absorption through the intestinal epithelial cell layer. This includes mechanical work (chewing, mixing and movement of food throught the digestive tract) and chemical degradation (proteolytic enzymes). Amino acids primarily act as molecular building blocks to ensure proper growth and development and for the maintenance of cells in the body. In addition, amino acids can be used as energy by catabolism when there is an excess of proteins or not enough carbohydrates or lipids to provide energy. An overview of the human digestive system is depicted in figure 1 and figure 2 shows the major steps in how proteins are digested and absorbed.

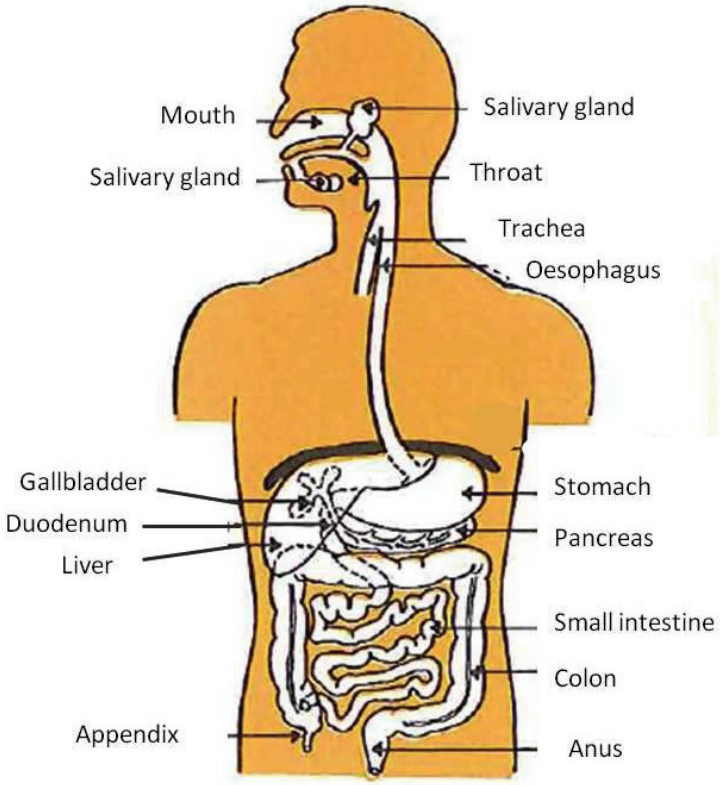


Figure 1. The human digestive system (modified from Nes et al., 1998 [1]).

Digestion of proteins begins in the mouth, where food is chewed and mixed with saliva before being swallowed. The proteolytic action starts in the stomach where gastric juice containing pepsinogen and hydrochloric acid (HCl) is secreted. Gastric juice is a highly acidic solution of pH 1.0-3.5 [2] which activates pepsinogen to form the active enzyme pepsin. In addition, HCl denatures some, but not all proteins thereby facilitating the

proteolytic action of pepsins by exposing previously hidden parts of the protein [3, 4]. The semi-digested proteins are gradually transferred to the duodenum through the pylorus. In the duodenum, pancreatic juice and bile are secreted through a common duct and these alkaline solutions neutralise the acidic gastric digesta. The collective action of the proteolytic enzymes in the pancreatic juice (trypsin, chymotrypsin, elastase and carboxypeptidases) and the bile salt secreted in the bile continues the proteolysis. All the duodenal proteases are released as inactive pro-enzymes and their activation will be described in the following section. In the epithelial brush border there are peptidases that further hydrolyse polypeptides as well as peptide and amino acid transport systems for absorption of degraded products. The final step in protein digestion is the action of intracellular peptidases degrading peptides to amino acids that are subsequently transferred to the blood. It has been reported that some intact dietary proteins or partially degraded polypeptides could be absorbed through transcytosis [5, 6]. However, very little protein is thought to cross the epithelia as the cell layer is equipped with numerous barriers to avoid such permeability [7].

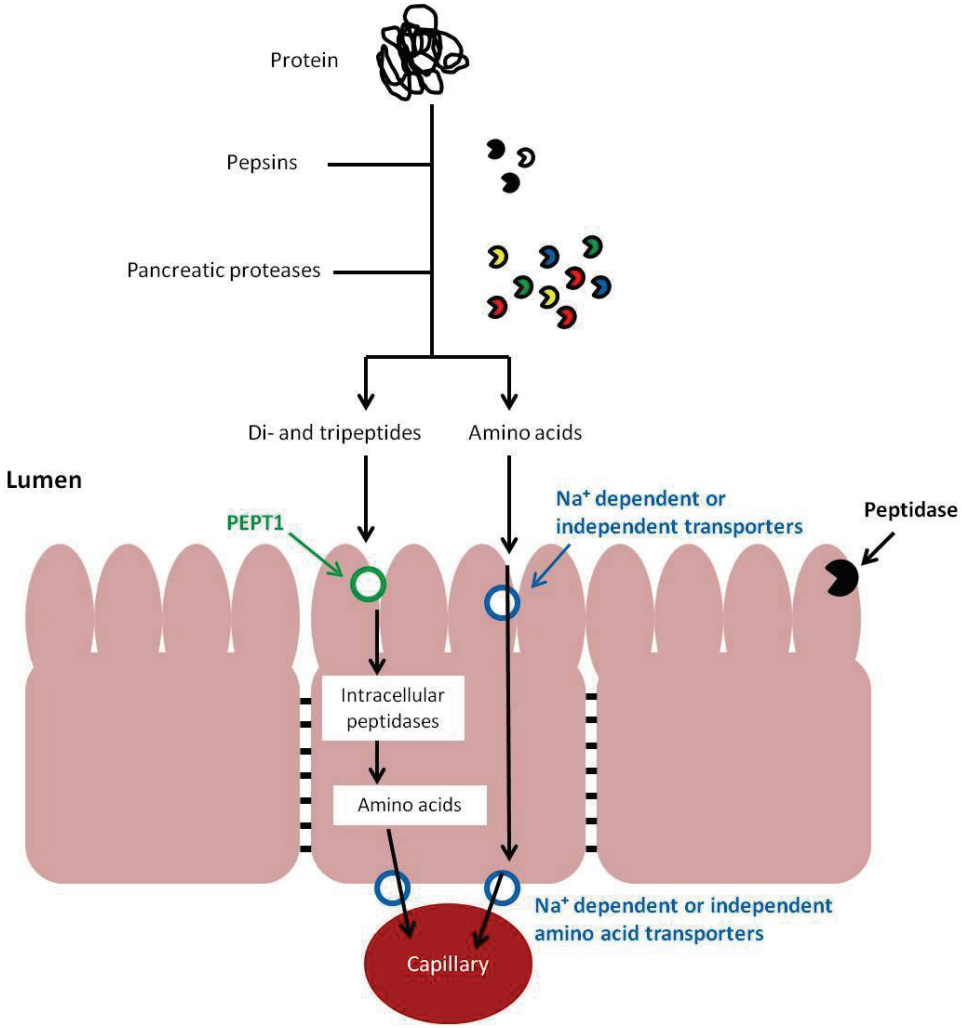


Figure 2. Digestion and absorption of proteins showing important enzymes and transporters. PEPT1, di/tripeptide transporter (modified from Goodman, 2010 [8]).

The nature of the food ingested (liquid or solid) as well as its components (lipid, carbohydrate and protein) and its overall caloric content affects the degree and the duration of the digestive enzyme response [9]. It appears that a meal containing 500 kcal is sufficient to ensure a maximal enzyme response and higher enzyme outputs are observed for high fat diets compared to carbohydrate rich diets. A solid meal is released more slowly from the stomach compared to a homogenised meal of the same composition and this prolongs the stimulation of the enzyme response. In addition, the volume, osmolarity and temperature of the meal will influence the secretion of digestive enzymes [9].

1.1.1 Gastric digestion

The arrival of food in the stomach stimulates the secretion of gastric juice by stretching the stomach and raising the pH of the stomach contents depending on the buffering capacity of the food [10]. Nerve reflexes are thereby activated which further leads to the secretion of stimulating hormones (acetylcholine, gastrin and histamine). Approximately 0.7 L gastric juice is secreted after a typical meal and 2 L is secreted per day [4].

The stomach functions mainly as a food storage organ and holds about 1-1.5 L after a meal. It is divided into four regions; the cardiac region, the fundic region, the body and the pyloric region (figure 3).

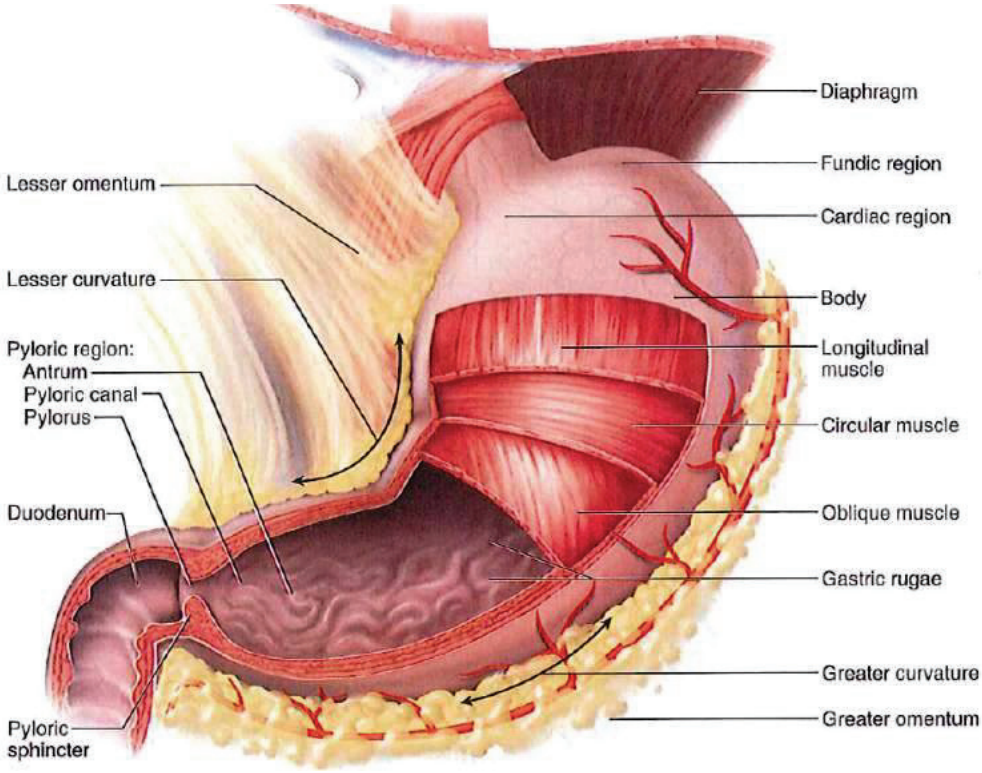
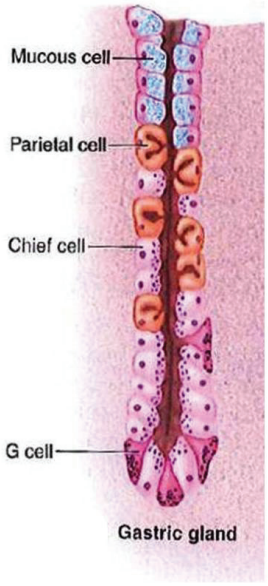


Figure 3. Anatomy of the stomach (from Saladin, 2007 [3]).

Gastric juice

Gastric glands are distributed throughout the stomach and are composed of various cell types that each has a specific secretory function (figure 4). Mucous cells secrete mucus that protects the stomach lining from HCl and the digestion by pepsin. Stem cells are found near the bottom and the neck of the glands and ensure a rapid regeneration of all cell types. Parietal cells secrete HCl and intrinsic factor, while the chief cells secrete pepsinogen. Finally, the enteroendocrine cells secrete hormones and paracrine messengers (eg: secretin, cholecystokinin, gastrin and histamine) that regulate digestion. Gastric juice is a solution composed of all these secretions [3].



Cells of the stomach	Function
Stem cells	Regenerate cells
Mucous cells	Secrete mucin
Parietal cells	Secrete HCL and intrinsic factor
Chief cells	Secrete pepsinogen
Enteroendocrine cells	Secrete hormones

Figure 4. The location of different cells in the gastric glands and their function (modified from Saladin, 2007 [3]).

Pepsin

The acidic stomach environment removes an N-terminal propeptide, covering the active site cleft, from pepsinogen to produce the active form, pepsin. This occurs by a local conformational denaturation of a part of the propeptide which next binds to the active site and is cleaved [11]. Pepsins are autocatalytic enzymes and will thereby activate more pepsin. These aspartic proteases have broad specificities and cleave proteins preferentially at sites involving hydrophobic and aromatic residues; phenylalanine, tyrosine, leucine, valine or methionine, either in combination or close to the bond cleaved [12].

Human gastric juice contains a combination of different pepsin isoforms. Pepsin A (3a, b, c and 1) constitutes about 90%, whereas 10% of the proteases in non-stimulated human gastric juice is found to be gastricsin (pepsin C) [13]. The occurrence of different pepsin variants is frequent also within other species [14]. For instance, in pig stomach a variant called pepsin B is present [11]. Human and porcine pepsin A has been shown to have 84% similarity [15]. Pepsins from different species may also exhibit different activities towards the same substrate as shown by Crévieu-Gabriel *et al*. [16].

The activity of the pepsins is highly influenced by pH. Different pepsins have different pH optima depending on the substrate used, and the proteolytic activity of human gastric juice is highly effective up to pH 4.0 [17]. Pepsin A displays a pH range of hydrolysis from below 1 to around 6 [11]. For hydrolysis of hemoglobin, pepsin A has a pH optimum near 2 while gastricsin displays a pH optimum near 3 [11]. For individuals treated for gastro-oesophageal reflux disease, gastric pH is elevated to >4 due to medication by proton pump inhibitors [18]. Gastric pH could also for a period of time be increased by the ingestion of food [19]. Elevated pH leading to low activity of the pepsins reduces gastric protein digestion, resulting in an increased amount of intact protein reaching the intestine [20, 21]

1.1.2 Intestinal digestion

Approximately 10-15% of the dietary proteins are partially digested by pepsins to polypeptides and a small amount of free amino acids. Most protein digestion however, occurs in the small intestine [8].

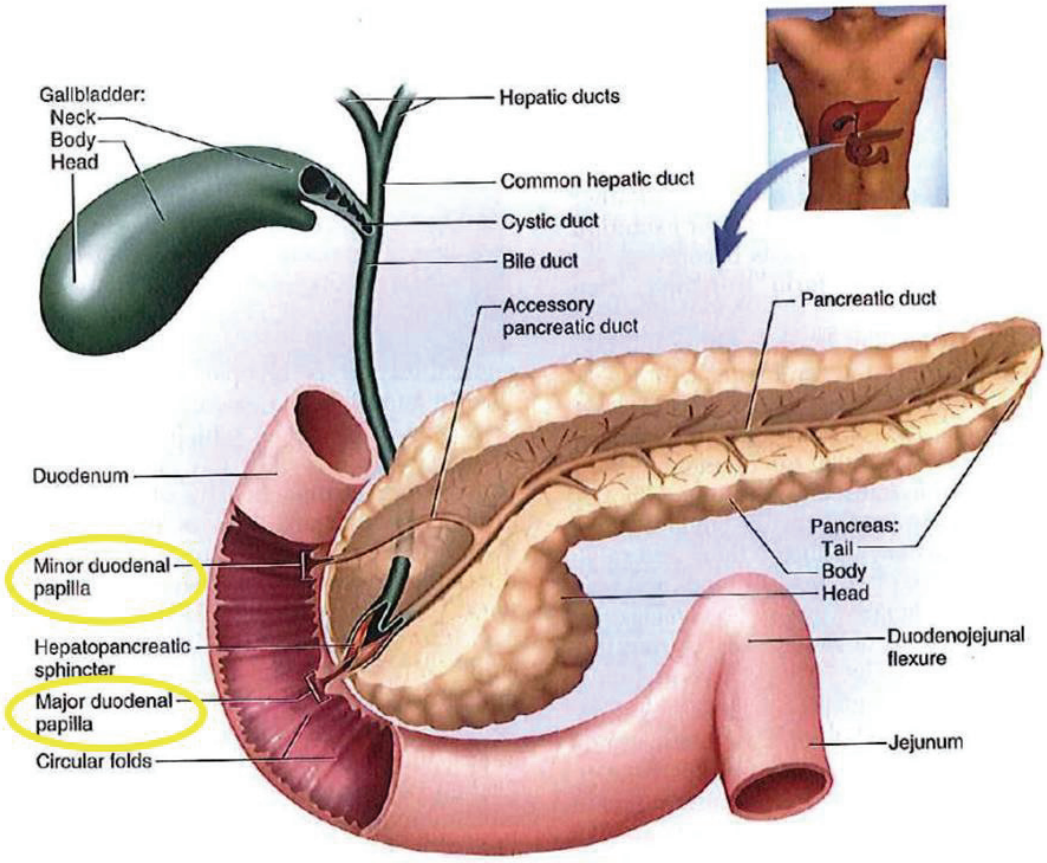


Figure 5. Anatomy of the duodenum, pancreas, gallbladder and bile passages. The yellow circles show where pancreatic juice and bile are secreted. Bile is always secreted through the major duodenal papilla with the pancreatic juice, while small amounts of pancreatic juice could also be secreted alone through the minor duodenal papilla (modified from Saladin, 2007 [3]).

Duodenal juice

Partially digested proteins are gradually transferred from the stomach by peristalsis to the duodenum, the upper part of the intestine. Here the pepsins are inactivated by the elevated pH of the duodenal juice. During duodenal digestion, the pH is normally about 6.2–6.7 [22, 23] however, pH values up to 7–8 are recorded both in *in vivo* and *in vitro* studies [24, 25].

Duodenal juice is the collective secretions of the duodenal epithelial cells, bile and pancreatic juice. Bile and pancreatic juice are emptied together into the duodenum through the major duodenal papilla, but pancreatic juice could also be secreted independently of bile through the minor duodenal papilla (figure 5).

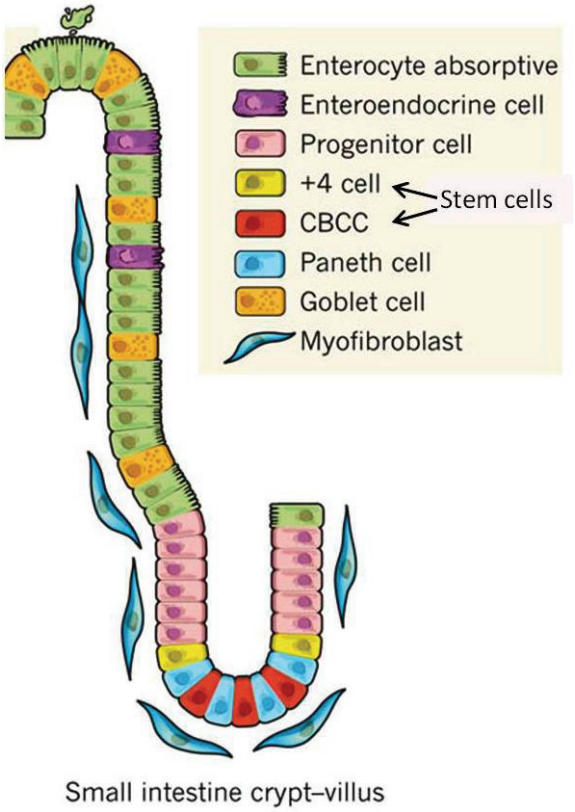


Figure 6. Organisation of the cells in the human small intestinal crypt-villus. Stem cells are located near the bottom of the crypt either between the Paneth cells or directly above these. Paneth cells move down to the crypt bottom and live longer than the other cell types that move up and are shed within a few days. CBCC-crypt base columnar cells (modified from Medema & Vermeulen, 2011 [26]).

The intestinal mucosa produces approximately 1 to 2 L of intestinal juice every day and it consists of five major cell types (figure 6) [1, 4]. The absorptive cells produce various peptidases that stay associated with the epithelial surfaces. In addition, these cells absorb amino acids as well as di- and tripeptides through specific transporter systems [27]. Cytoplasmic extensions called microvilli on the surface of all absorptive epithelial cells form the brush border. Goblet cells produce mucus to protect the epithelia from the actions of digestive enzymes and the acidic gastric digesta. Paneth's cells contain granules of antimicrobial molecules such as defensins and lysozyme that are secreted to

protect against invading microorganisms [28, 29]. Hormones (secretin and cholecystokinin) on the other hand, are produced by the enteroendocrine cells to regulate gastrointestinal secretions. All these cell types are frequently renewed by the intestinal stem cells residing near the bottom of intestinal glands.

The liver produces about 0.6-1.0 L of bile every day, and the gallbladder serves as a place to store and concentrate this fluid [1, 4]. Bile is a yellow-green liquid containing minerals, cholesterol, neutral fats, phospholipids, bile pigments (eg bilirubin) and bile acids [3, 30]. Human bile acids include cholic, deoxycholic and chenodeoxycholic acids forming bile salts when conjugated by taurine and glycine [31]. Bile salts emulsify fats and act as a cofactor for bile salt dependent lipase [30]. Besides their role during lipid digestion, bile salts have also by *in vitro* studies been shown to accelerate proteolysis [32].

About 1.2-1.5 L of pancreatic juice is secreted every day and it contains a mixture of enzymes, proenzymes, protease inhibitors, sodium bicarbonate and other electrolytes in water [1, 4]. The enzymes secreted in this juice are pancreatic lipase and amylase as well as ribonuclease and deoxyribonuclease to digest fat, starch, RNA and DNA, respectively. The proteolytic enzymes however, are released as inactive proenzymes to avoid self-digestion. They are called trypsinogen, chymotrypsinogen, proelastase and procarboxypeptidase. Activation of these proenzymes takes place when they are released into the intestinal lumen and their activation peptides are cleaved off [33]. Trypsinogen is first activated by enteropeptidase (enterokinase) residing on the duodenal brush border, producing the active enzyme trypsin. Trypsin further activates chymotrypsinogen, proelastase and procarboxypeptidase to produce chymotrypsin, elastase and carboxypeptidase, respectively. In addition, trypsin also activates more trypsin. Sodium bicarbonate, in both the pancreatic juice and bile, buffers the HCl arriving from the stomach .The elevated duodenal pH is also more optimal for the activity of the pancreatic proteases [3].

The secretion of human pancreatic fluid is cyclical in the fasting state, depending on the motility of the upper gastrointestinal tract [9]. After ingestion of a food however, the delivery of enzymes into the duodenum increases rapidly and within an hour maximal values are obtained. Cholecystokinin, secretin and vagal nerve stimulation increases the release of bile and the secretion of enzyme-rich pancreatic juice. Pancreatic enzymes are generally secreted in parallel with postprandial lipase:amylase and lipase:trypsin ratios being approximately 3–6/1 and 5–10/1, respectively [9].

Duodenal proteases

The duodenal proteases include the pancreatic enzymes trypsin, chymotrypsin, elastase and carboxypeptidase synthesized by acinar cells and secreted as proenzymes in addition to the amino- and dipeptide peptidases produced by the epithelial cells. Trypsin, chymotrypsin and elastase are all endopeptidases of the serine-protease family having serine residues in their active sites. The carboxypeptidases, on the other hand, are metalloproteinases that need zinc ions in their active sites to exert their catalytic activities [33].

Trypsin

Trypsin is the most abundant of the pancreatic digestive enzymes, making up around 19% of the total pancreatic juice protein [11]. The enzyme is essential during the activation of all the other pancreatic proteases as well as itself. Three major trypsin isoforms are active within the human duodenum and they all hydrolyze peptides at the carboxyl side of lysine and arginine. If, however, a proline residue is present at the carboxyl side of the cleavage site, no cleavage will occur. The three isoforms have similar protein structure with the active proteins composed of 174 amino acids, a 14-amino acid signal peptide, and an 8-amino acid activation peptide [33]. Two-thirds of the trypsin activity is made up of the *cationic trypsin* whereas about one-third is made up the *anionic trypsin*. The third isoform, representing less than 5% of the total trypsin activity, is called *mesotrypsin*. Mesotrypsin is resistant to the pancreatic secretory trypsin inhibitor, a specific inhibitor of the cationic and anionic trypsins [34]. A fourth trypsin-like enzyme, called *pancreasin* has also been identified but little is known about its function [35]. Trypsins have optimal activity between pH 7.5 and 8.5 in the presence of Ca^{2+} [11, 33]. Human and porcine cationic trypsin has about 80% sequence similarity (http://blast.ncbi.nlm.nih.gov).

Chymotrypsin

After activation by trypsin, chymotrypsin hydrolyses peptides at the carboxylic side of the aromatic amino acids (phenylalanine, tyrosine, and tryptophan). About 9% of the total protein of pancreatic juice is made up of this enzyme which makes chymotrypsin the second most abundant of the serine proteases [33]. The active enzyme has 230 amino acids and the proenzyme has an additional signal peptide of 18 amino acid residues and an activation peptide of 15 amino acids. Chymotrypsin has optimum activity in the pH range 5-9 [36].

Three isoforms of human pancreatic chymotrypsin, A, B and C have been identified [36-38]. Chymotrypsin A accounts for most of the activity and chymotrypsin B is the second most active of the enzymes. Human chymotrypsin C has recently been shown to function as a specific regulator of the activation and degradation of human cationic trypsinogen and trypsin. In addition, this enzyme is a co-activator of the procarboxypeptidases A1 and A2 [36, 38]. This isoform shows high sequence identity with the pancreatic elastases but demonstrates chymotrypsin-like substrate specificity as it

cleaves after phenylalanine, tyrosine, leucine, methionine, asparagine and glutamine residues [38].

Table 1. Overview of human proteases from the stomach and the pancreas (from Barrett *et al*., 2004, Roberts, 2006, Whitcomb & Lowe, 2007, Szmola *et al*., 2011 and Goodman, 2010 [8, 11, 33, 38]).

Enzyme	Action	Products
Pepsin Pepsin A (pepsin 1, 2, 3a,3b, 3c, 4?) Gastricsin (pepsin 5, 6 or pepsin C) Cathepsin (pepsin 7)	Broad specificities; cleave proteins preferentially at sites involving hydrophobic and aromatic residues	Polypeptides
Trypsin Cationic trypsin (PRSS1) Anionic trypsin (PRSS2) Mesotrypsin (PRSS3) Pancreasin (?)	Endopeptidases; Cleaves internal bonds at carboxyl side of lysine or arginine residues; activates other pancreatic proenzymes Cleaves preferentially at the carboxyl side of arginine	Oligopeptides
Chymotrypsin Chymotrypsin A Chymotrypsinogen B1 Chymotrypsin-like protease Chymotrypsin C	Endopeptidases; cleaves bonds at the carboxyl side of phenylalanine, tyrosine, leucine, and tryptophane residues High activity for cleavage of leucyl bonds	Oligopeptides
Elastase (unclear which form is secreted) Elastase 2A, 2B Elastase 3A, 3B	Endopeptidases; cleaves bonds after small amino acid residues – alanine, glycine, serine	Oligopeptides
Carboxypeptidase A A1, A2, A3	Exopeptidase; cleaves aromatic, neutral, or acidic amino acids from carboxyl terminal end of proteins and peptides	Aromatic amino acids and
Carboxypeptidase B B1, B2	Exopeptidase; cleaves arginine or lysine from carboxyl terminal end of proteins and peptides	Arginine, lysine, and peptides

Elastase

Pancreatic elastase splits the protein backbone at bonds of uncharged small amino acids (such as alanine, glycine, and serine) after activation by trypsin. It is the only enzyme able to digest elastin. The sequence of human elastase has recently been identified

through the human genome project, and two forms have been identified. It is, however, unclear which form of elastase is secreted (2A, 2B, 3A or 3B) [33]. Elastase is active in the pH range 7.5-10.5 [36].

Carboxypeptidase A and B

Trypsin also activates procarboxypeptidases to create a group of enzymes called the carboxypeptidases. These enzymes are exopeptidases by cleaving proteins and peptides one amino acid at a time from the carboxyl end. Carboxypeptidase A (CPA) cleaves off the last C-terminal amino acid of a target peptide chain when it is aromatic, neutral, or acidic, while carboxypeptidase B (CPB) cleaves off the last carboxylic amino acid when it is basic (arginine or lysine). This indicates that CPA cleaves peptides after initial chymotrypsin cleavage, while CPB hydrolyses previously trypsin-cleaved peptides.Three forms of carboxypeptidase A are found in humans. Carboxypeptidase A1 and A2 are secreted as monomers, while carboxypeptidase A3 form a complex with elastase [33, 36]. The activity of carboxypeptidases A1 and A2 is increased by the action of chymotrypsin C [38]. Carboxypeptidase B is found in two forms in humans called B1 and B2 [11, 33, 36].

Intestinal peptidases

The intestinal cells produce various peptidases that stay associated with the brush border to complete the hydrolysis of polypeptides to di- and tripeptides and amino acids. These peptidases include aminopeptidases that remove amino acids one by one from the amino-terminal, dipeptidases cleaving dipeptides to release two free amino acids and dipeptidyl amino- and carboxypeptidases releasing dipeptides from the amino and carboxyl side of peptides [39].

Absorption of protein degradation products

The small intestine is composed of three sections. The duodenum is the first 25 cm, the jejunum is approximately 2.5 m long and the ileum is about 3.5 m long [4]. Nutrient absorption occurs mainly in the duodenum and jejunum. The epithelial cells in the colon are also able to absorb digested protein. The colon is, however, likely to predominantly be involved in the transport of digested bacterial protein since the small intestinal transport is very efficient [27]. The intestinal mucosa is folded and has fingerlike projections called villi, each connected to blood and lymph through capillary networks. Together with the microvilli forming the brush border on the epithelia, this results in a very large surface area for nutrient absorption.

In the intestinal lumen, the end products of protein digestion are a mixture of free amino acids and short peptides (up to 80%). All di- and tripeptides are transported into the intestinal epithelial cells via one specific peptide transport system called the peptide transporter 1 (PEPT1) [8, 27, 40]. This transport is H^+-dependant, taking advantage of the about ten-fold higher concentration of H^+ ions in the intestinal lumen compared to the intracellular environment. PEPT1 is found mainly in the duodenum and jejunum and this is therefore where the absorptive capacity for peptides is the highest [27]. The

intestinal absorption of amino acids on the other hand, is accomplished by several somewhat overlapping transport systems with broad specificities (Table 2). The transport mechanism used as well as the type of amino acid transported (acidic, basic, neutral and zwitterionic) define these tranporters [8]. Amino acid transport is performed by either active transport or facilitated diffusion. The active transport systems in the intestinal brush border and the basolateral membrane use adenosine triphosphate (ATP) as energy and the driving forces are transmembrane ion gradients and membrane potential [27].

Table 2. Some common amino acid and peptide transporters in intestinal epithelial cells. The classification system uses uppercase letters to identify Na^+ gradient-dependent transport systems and lowercase if independant of Na^+. The electrical nature of the amino acid substrate recognised by the transporters are indicated by superscripts such as "0", "+" and "-". Glu = glutamic acid, Asp = aspartic acid (modified from Ganapathy *et al*., 2006 and Goodman, 2010 [8, 27]).

Transport system	Amino acid substrate	Cotransported ions	Type of transport
Apical transport			
B^0	Neutral	Na^+	Secondary active
B^{0+}	Neutral, basic and cystine	Na+ and Cl^-	Secondary active
b^{0+}	Neutral, basic and cystine	None	Exchanging*
Y^+	Basic	None	Facilitated diffusion
Imino	Imino	Na^+ and Cl^-	Secondary active
X_{AG-}	Acidic (only Glu and Asp)	Na^+, H^+ and K^+	Secondary active
β	β	Na^+ and Cl^-	Secondary active
PAT1	Imino	H^+	Secondary active
ASC	Neutral with 3-4 carbons	Na^+	Exchanging**
PEPT1	Di- and tripeptides	H^+	Secondary active
Basolateral transport			
A	Neutral and imino	Na^+	Secondary active
Asc	Neutral with 3-4 carbons	None	Facilitated diffusion
L	Neutral, large and hydrophobic	None	Facilitated diffusion
y^+	Basic	None	Facilitated diffusion

*The absorption of basic amino acids or cystine is coupled to the release of neutral amino acids

**The absorption of a neutral amino acid into the cell is coupled to the release of another neutral amino acid out of the cell

Glu = glutamic acid, Asp = aspartic acid

Considerably more amino acids enter the cells as di- and tripeptides than what enters as single amino acids. Inside the cells dipeptidases and tripeptidases cleave most of the peptides to their component amino acids. Some peptides containing proline and hydroxyproline are quite resistant to cleavage by the cellular peptidases and are transferred to blood by a basolateral peptide transporter [27]. Glutathione (γ-Glu-Cys-Gly) is an important antioxidant peptide protecting the cell membrane lipids from oxidation by free radicals. It is absorbed intact and has its own transport systems in both the apical and basolateral membrane [27]. Also, a small amount of the antihypertensive tripeptides Val-Pro-Pro has been shown to be transported intact through a monolayer of the human colon adenocarcinoma cell line Caco-2 [41, 42]. The main mechanism behind the transport of this peptide was suggested to be paracellular diffusion.
Amino acids leave the epithelial cells through basolateral transport systems (Table 2). Some of these transporters are responsible for the release of amino acids from the cell to the bloodstream by Na^+ independent facilitated diffusion. Other Na^+ dependent transport systems provide the intestinal cells with amino acids from the blood during periods between meals [8, 27]. The amino acids are further transported through the hepatic portal vein to the liver via the blood capillaries connected to the villi. The fate of the amino acids is dependant on the requirements of the body. They could be modified in the liver or distributed to other parts of the body through the bloodstream.

1.1.3 *In vitro* model systems to study digestion

Human intervention studies are time consuming and expensive due to the number of people that need to be involved (hospital staff, researchers and volunteers). In addition, *in vivo* experiments are limited by ethical considerations and by the difficulty in extracting representative samples from the digestive tract via narrow aspiration tubes. Furthermore, animal studies are also frequently considered irrelevant to humans [43]. For this reason, human digestion is frequently mimicked *in vitro* by researchers all over the world.

In vitro digestion models can be rapid, low cost and may offer the possibility to measure the digestion of solid food that is difficult to study *in vivo*. Often the digestive process is studied in several stages, each simulating the mouth, the stomach, the small intestine or the colon. To account for the absorption occuring *in vivo*, the step simulating digestion in the small intestine is frequently coupled to dialysis membranes with low molecular weight cut-off [44-46]. Another means to study absorption is by using Caco-2 cells cultured on semipermeable membranes (figure 7) since these cells undergo spontaneous enterocyte differentiation in culture [47]. After culturing Caco-2 cells for 2-3 weeks they form monolayers with tight junctions and microvilli at their apical surfaces. In addition, they express many brush border digestive enzymes (peptidases), transporters (PEPT1 and amino acid transporters) and receptors [41, 42]. The products of *in vitro* gastric and

duodenal digestion may be added to the apical side and the apical and basolateral media analysed after a period of time.

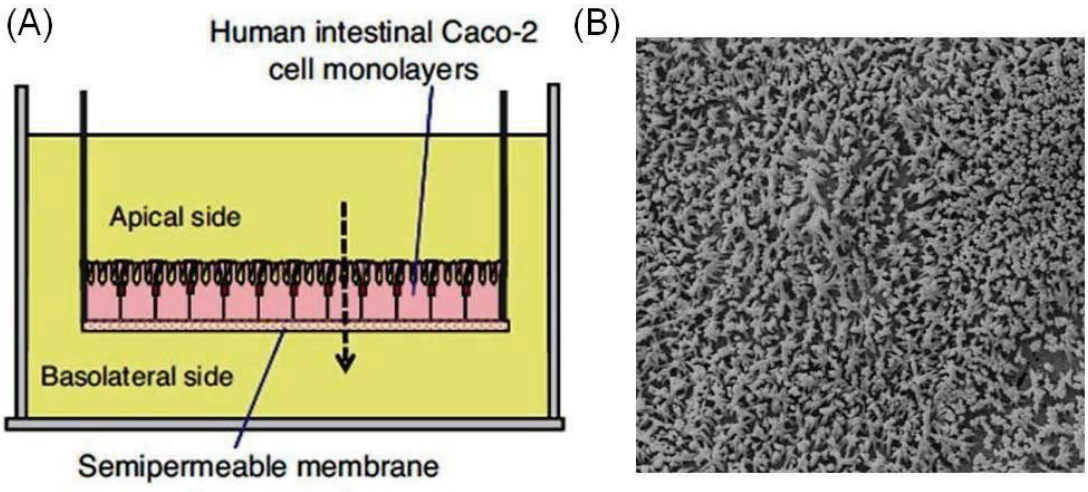


Figure 7. (A) Caco-2 cells cultured *in vitro* on semipermeable membranes form monolayers. (B) Scanning electron microscopy picture of the apical brush border on Caco-2 cells cultured for 26 days on a semipermeable membrane in our laboratory ((A) from Shimizu, 2010 [42] and (B) picture taken by Elin Ørmen at the UMB Microscopy Lab).

Digestion models can be either static or dynamic. Static models use simple 37 °C incubation of food for a certain amount of time at average pH-values reported for the different parts of the body normally using either purified commercial enzymes or mixtures of such. Samples are mixed by magnetic stirring or shaking and there is no removal of the digestion products during the process. The loss or appearance of components such as proteins or peptides is frequently used to evaluate the digestive process [43]. Dynamic models on the other hand, take advantage of sophisticated computer-controlled pump-driven simulation of gastrointestinal churning and movement of food through separated compartments simulating the stomach and intestine. Often products of digestion are also removed by dialysis to simulate intestinal absorbtion [45]. The adjustment of pH and addition of simulated gastric and intestinal juices in these models is validated by comparing *in vitro* and *in vivo* results [43]. Examples of such dynamic models include the TNO gastrointestinal tract model (TIM; Zeist, The Netherlands) [48] and the dynamic gastric model and small intestinal simulation developed at the Institute of Food Research (Norwich, UK)[43]. The dynamic digestion models are under continuous development as illustrated by the newly developed 3D gastric model characterising movements of the gastric contents by computational fluid dynamic techniques [49, 50].

Most digestion models have so far used highly purified commercial enzymes of non-human origin, often porcine or bovine pepsin, trypsin and or chymotrypsin [51-55]. In human digestive juices, however, various isoforms of the proteolytic enzymes are present [13] and these isoforms could differ from those found in other species. Different

enzyme isoforms could also have different catalytic acitivity depending on the substrate and the pH during digestion [15]. Mixtures of different isoforms as in the human digestive fluids could therefore give rise to other peptides when used to simulate digestion compared to the use of purified enzymes. In addition, digestion is influenced by other molecules such as bile acids, phospholipids and enzyme inhibitors that are present in normal duodenal juice [32, 51]. The contribution by such substances to the final digestive product is neglected if purified commercial enzymes are used.

Ongoing research, especially in the field of testing drugs for their absorption in the gastrointestinal tract, aims to completely characterise the composition of gastric and intestinal juices. Standard dissolution medias are made according to the current knowledge of the composition of the digestive fluids [53, 56-58]. Simulated gastric (SGF) and intestinal (SIF) fluids have been developed for both fasting and fed states [51, 53, 56, 59, 60]. A recent review on *in vitro* human digestion models by Hur *et al*. [61] demonstrates the vast variability of digestive fluids used, and emphasises the importance of using physiologically relevant levels of enzymes and other minor components when designing these fluids. However, all physiological mechanisms operative *in vivo* are not transplantable to any *in vitro* setting and no model can internalize all potential individual variability in enzyme secretion created by hormonal, peptide and neural regulation.

1.2 IMMUNOLOGY OF THE GUT

Immunity means the ability of an organism to resist infection. The immune system operates in the entire body and consists of a number of different molecules, cells and organs that collectively work to protect their host from potentially harmful microorganisms, and from the development and growth of cancer cells. A circulatory system of lymphatic vessels and lymph nodes in close contact with the blood circulation connects the different lymphoid organs. Immune cells monitor the state of infection in the body by circulating in the blood and lymph systems.

1.2.1 Innate and adaptive immunity

The immune system is divided into the innate and the adaptive immune system which differ with regard to pathogen recognition and removal. Innate immune cells include phagocytosing cells such as macrophages, monocytes, granulocytes and dendritic cells (DC) as well as mast cells and natural killer cells. In addition, the innate immune system is made up of physical barriers such as the epithelia and soluble molecules such as the complement system, cytokines and antimicrobial proteins and peptides. The innate immune system rapidly acts against potentially harmful intruding microorganisms by a

non-specific recognition of conserved structures for several pathogens. This non-specific recognition is necessary to keep invading organisms at a low number before the specific immune response to a certain antigen is developed.
Adaptive immunity, in contrast, relies on recognition of specific structures unique for a certain pathogen. The activation of the adaptive immune cells, the T and B lymphocytes, is initiated through a close cooperation with the innate immune system. While native antigens are recognised by immunoglobulin receptors on B lymphocytes, short peptide fragments are recognised by the T cell receptor (TCR). Activation of T cells requires close contact with professional antigen presenting cells (APCs) such as DCs. DCs recognise antigen structure through their Toll-like receptor, engulf antigens, digest them intracellularly and present peptide fragments in their major histocompatibility complex (MHC) molecules. The primary T cell activation signal is provided when the T cell receptor binds to the MHC-peptide complex. Activated lymphocytes develop into effector cells, however, this process takes time. The adaptive immune system against a pathogen encountered for the first time is therefore often not effective for up to a week after infection. Upon infection for the second time by a known pathogen, the adaptive immunity will respond faster and more effectively. This feature is called immunological memory and it is characteristic of the adaptive immune system.

Immunological tolerance, the inability to elicit an immune response to certain antigens, is another important feature of the lymphocytes. Tolerance is necessary for the body to distinguish between self and non-self. Tolerance occurs when the lymphocytes are exposed to antigens during their maturation in the bone marrow and the thymus and learn not to recognise host (self) antigens. If this process fails, the immune cells can start attacking host antigens and autoimmune diseases might arise. Lymphocytes also exhibit oral tolerance, unresponsiveness towards most food components. This is of course important to ensure a safe passage of food through the digestive tract and thereby avoid food allergy [(62)].

1.2.2 Activation of T lymphocytes

T lymphocytes are important for the regulation and coordination of the overall immune response. They are specialised to act against infected cells, and are therefore dependent on other cells to inform them on what is in their cell interior. T lymphocytes can develop into two main types of effector cells, the T helper cells (Th) or the T cytotoxic cells (Tc). Tc cells are $CD8^+$ and they interact and destroy antigen-bearing cells directly. Th helper cells, on the other hand, are $CD4^+$ and secrete signalling molecules called cytokines after interaction with antigens.

T cells can be activated by interact with peptides presented to them in MHC molecules on APCs. MHC class I molecules are attached to nearly all the cells in the body, whereas MHC class II molecules limit their expression to B cells, dendritic cells,

monocytes and macrophages, all of which are professional APCs. $CD4^+$ Th cells recognise antigen peptides in combination with MHC class II molecules, while $CD8^+$ Tc cells react with MHC class I molecules in combination with foreign peptides [63].

When a TCR interacts with an antigen presented by an APC, a transmembrane complex of polypeptides called the CD3-complex, transfers activation signals to the inside of the T cell. This signal is referred to as *signal 1*.

Other accessory membrane molecules on the T cells contribute to the activation process by binding to corresponding structures on the surface of cooperating cells. CD4 and CD8 function as receptors for MHC class II and MHC class I, respectively [63].
CD28 is another T cell surface molecule. It interacts with a corresponding structure called B7 (CD86/CD80) in the APC membrane, to send a second signal to the T-cells interior. Both signal 1 and 2 are essential for the T cells to develop into effector cells [64].

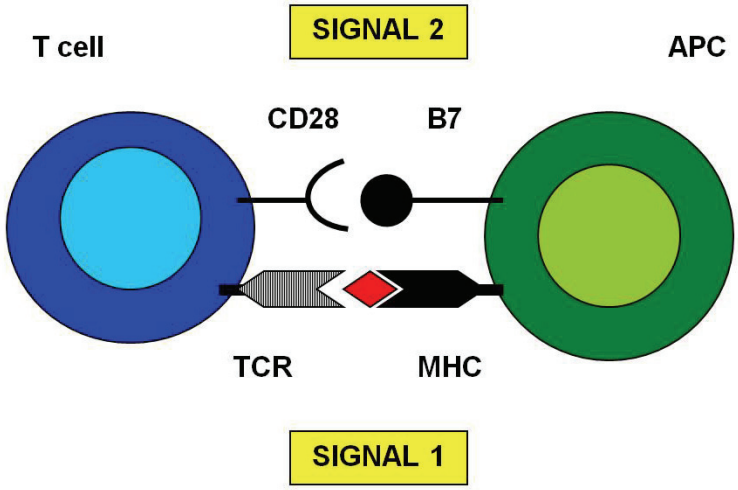


Figure 8. Activation of T lymphocytes mediated by two activation signals.

Shortly after activation, the T cell increase in size to form a so-called blast cell. Next, the blast cell starts expressing CD25, an activation antigen part of the high affinity interleukin (IL)-2 membrane receptor (IL-2R). Next the T-cell blast produces and releases the cytokine interleukin-2 (IL-2). Binding of IL-2 to its receptor induces T lymphocyte cell division [63].

1.2.3 The role of cytokines in immunity

Cytokines are low molecular weight signalling molecules secreted by both immune and non-immune cells to regulate the immunity. They act by binding to specific receptors located on the cytokine producing cell or on cells close by [65]. Cytokines can have partly overlapping effects and they can either strengthen or inhibit each others separate effects. Different cells produce and react to different cytokines and the secreted

cytokines surrounding naïve T cells are essential during their differentiation into the different effector cells.

T helper cells ($CD4^+$) are divided into two main subsets based on their secretion of cytokines. T helper 1 (Th1) cells are primarily involved in immunity against intracellular pathogens and secrete IL-2, IL-12 and IFN-γ. T helper 2 (Th2) cells on the other hand, produce IL-2, IL-4, IL-5, IL-6, IL-9, IL-13 and IL-10 to promote the proliferation of B cells and their antibody secretion [66, 67]. It has been shown that Th1 and Th2 cells can cross-regulate each others development by the secretion of cytokines. The Th1 cytokine IFN-γ could inhibit the development of Th2 cells while promoting Th1 differentiation. Likewise, Th2 cell secrete IL-4 which inhibits differentiation into Th1 cells. The Th1 vs Th2 classification is, however, likely an oversimplification of events since some immune responses requires both Th1 and Th2 cells [66].

An additional T-cell subset called Th17 has also been identified. Th17 cells secrete neither IL-4 nor IFN-γ, but rather IL-17 [68].

Several regulatory T cells have also been identified including Th3, Tr1 and CD4+CD25+ cells. Th3 cells produce the anti-inflammatory cytokine tumour growth factor (TGF)-β and various amounts of IL-4 and IL-10. Tr1 cells produce another anti-inflammatory cytokine, IL-10, which also drives the generation of these cells. Finally, the immunosuppression by CD4+CD25+ regulatory T cells is mediated by TGF-β bound to their surfaces. The latter cells also express the transcription factor forkhead box P3 thought to block both Th1 and Th2 responses [62].

1.2.4 Intestinal immunity

The mucosal barrier

The mucosal surface of the stomach and the intestines is covered by a simple layer of epithelium. To avoid the entry of macromolecules and microorganisms across this monolayer, the space between the cells is sealed by protein strands called tight junctions [69]. In addition, the brush border on the intestinal epithelium is coated by mucus produced by the goblet cells and mucin-like glycoproteins called the glycocalyx providing protective barriers against these unwanted substances [7, 70]. Paneth cells also produce an array of antimicrobial substances such as defensins (lyses microbial membranes by forming pores), lactoferrin (chelates iron that bacteria need to grow), peroxidase (produces free radicals) and lysozyme (digests the coat of Gram-negative enterobacteria) [7, 71]. The epithelial cells are furthermore rapidly and contiuosly renewed by pluripotent stem cells located near the bottom of the intestinal crypts. This ensures that cells damaged by harmful substances such as toxins and carcinogens are eliminated and replaced by healthy cells [7]. In addtion to the mentioned defense mechanisms, gut homeostasis is maintained by secreted antibodies (secretory IgA) as well as by the

induction of unresponsiveness towards unharmful substances such as food and commensal bacteria (oral tolerance) [72].

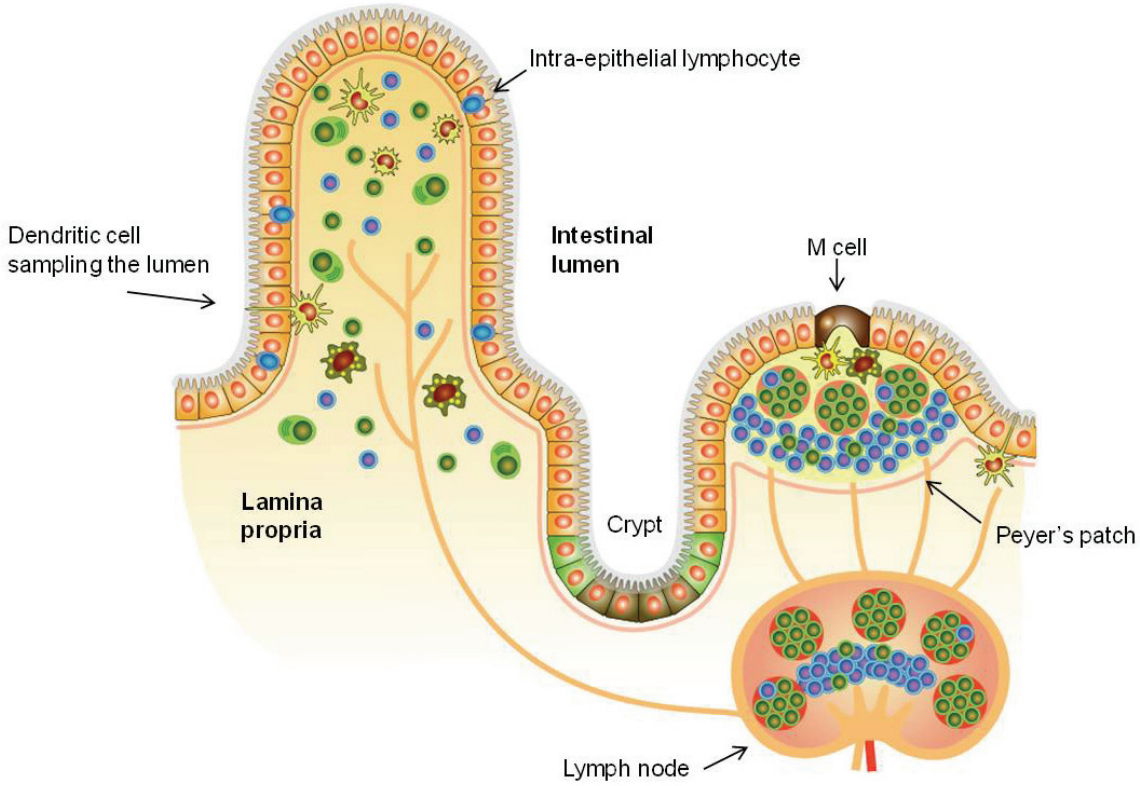


Figure 9. Organisation of the mucosal immune system. Antigens enter either through the M cells in the follicle-associated epithelium, by dendritic cells sampling the lumen or by the epithelial cells. Processed antigens are presented to T cells in the Peyer's patch or in the mesenteric lymph nodes. Blue and green cells represent T and B cells respectively, while yellow cells with projections and green cells with yellow granules symbolize dendritic cells and macrophages respectively (from Lea, 2011).

Associated with the intestinal epithelium there is also a specialised mucosal immune system as shown in figure 9. This immune system is divided into inductive and effector sites. Inductive immune sites include the mesenteric lymph nodes (MLN) and the gut-associated lymphoid tissue (GALT). The GALT is composed of Peyer's patches (PP), the appendix, and isolated lymphoid follicles (ILFs). The inductive sites are where naïve T and B cells are stimulated by antigens sampled from the mucosal surfaces. The effector sites on the other hand is where differentiated effector cells act, for instance by making sure that antibodies against the antigen is being produced. Effector sites include the lamina propria (LP), the connective tissue located just beneath the epithelial monolayer and the surface epithelia itself [72].

Anatomy and physiology of the GALT

In humans, PPs are mainly located in the distal ileum. They are specialised immune structures which contain between 5 and 200 aggregated lymphoid follicles. Lymphoid follicles are sites packed with B and T lymphocytes organised as large B cell follicles

and an interfollicular T cell area [73]. The epithelial cells overlaying the PPs are called the follicle associated epithelium (FAE). This part of the epithelium has fewer microvilli, produces less digestive enzymes and does not have a mucus layer [74]. Directly underneath the FAE is a region called the sub epithelial dome (SED) where immature DCs as well as macrophages are located [74]. *In vivo*, antigens such as food peptides are actively sampled from the intestinal lumen through transcytosis by microfold (M) cells located in the FAE. They transport peptides across the epithelium and transfer intact peptides to the many DCs located in the SED. DCs being professional antigen presenting cells (APCs), process and present these peptides to T cells in the Peyer's patches, or transport peptides through lymph to T cells in the mesenteric lymph nodes (MLN) [75].
The development of IgA^+ B cells is also taking place in the PPs. These cells migrate through the lymphatic system to the LP via the MLNs. In the LP B cells mature into plasma cells producing IgA which is secreted as a dimer into the intestinal lumen [62, 73]. Secretory IgA prevents the entry of potentially harmful substances into the epithelial cells and bind to microorganisms thereby neutralising these.

Peptides are also sampled from the intestinal lumen directly by DCs of the lamina propria extending their dendrites through the epithelial layer [76, 77]. The integrity of the epithelial barrier is not disrupted by these DCs since they express tight junction proteins on their surfaces [76].

Intestinal epithelial cells are associated with two main lymphocyte populations, the LP lymphocytes as well as the intra-epithelial lymphocytes (IELs), which are associated with the basolateral membrane of the IECs [78]. Intestinal epithelial cells are also able to process and present luminal antigens that have been sampled from the apical surface [62]. The processed antigens are presented to the associated lymphocytes by MHC class I or II molecules mostly expressed on the basolateral side of the cell. Local suppression of immune responses towards food antigens are induced by a selective activation of CD8+ suppressor T cells by the IECs [62]. Intestinal epithelial cell, however, lack the expression of the costimulatory molecules CD80 and CD86 and are thereby not able to contribute with the second T-cell activation signal. This could result in the induction of anergy in CD4+ T cells [78].

1.3 MILK PROTEINS

1.3.1 Milk

In the Western part of the world, including Norway, milk and dairy products are consumed by children and adults as part of their normal diet. Milk is primarily secreted to meet the species specific nutritional requirements of all mammalian neonates [79]. In addition, milk is rich in a number of substances that are intended to protect the newborn from potentially harmfull substances while the intestinal barrier and immune system is developing [80, 81]. This includes immunoglobulins, lactoferrin, oligosaccarides and free fatty acids [79, 81, 82]. Only humans consume milk after weaning and then the milk consumed comes from other species. There is an ongoing debate as to whether milk consumption by adults is solely good for the consumer. Lactose intolerance is widespread [83] and hypersensitivity towards milk proteins has been hypothesised to be the true cause of many previously unexplained gastrointestinal disorders [84]. Nevertheless, milk is considered a "complete" food because of its rich content of minerals and vitamins in addition to proteins, fat and carbohydrates. Another positive effect seem to be that women drinking milk during childhood and adolescence have increased bone mass and density in adulthood [85] possibly reducing their chances of developing osteoporosis [86]. A negative association between premenopausal breast cancer and milk consumption has also been shown in a Norwegian cohort study [87]. In addition, researchers have for more than a decade focussed on identifying possible bioactive peptides produced by *in vitro* digestion of milk proteins using commercial enzymes [88-92].

1.3.2 Proteins

Bovine and caprine milks have a protein content of about 3-3.5%, whereas human milk contains only about 0.9% protein [93, 94]. Milk proteins are divided into two groups, the caseins and the whey proteins of which the caseins make up around 80% of the total protein content. The caseins are defined as the proteins precipitating from milk by acidification to pH 4.6 at 20°C, while the whey proteins remain in solution [95]. The relative concentration of the caseins and whey proteins also differs between species. Bovine milk displays a casein:whey ration of 5 and human milk has a casein:whey ratio of 0.9. In caprine milk, the casein concentration varies greatly due to genetic polymorphism and the casein:whey ratio therefore varies from 5 to 7 [94, 96]. An overview of the proteins in bovine skim milk, their concentration, major genetic variants, molecular weights and isoelectric points is given in table 3.

Table 3. Proteins in bovine skim milk - their concentration, major genetic variants, molecular weights and isoelectric points (from Farrell *et al*., 2004 [97]).

Protein	Composition in skim milk (g/L)	Major genetic variants	Molecular weight*	Isoelectric point
α_{s1}-casein	12–15	B	23,615	4.44–4.76
		C	23,542	…
α_{s2}-casein	3–4	A	25,226	…
β-casein	9–11	A^1	24,023	…
		A^2	23,983	4.83–5.07
		B	24,092	—
κ-casein	2–4	A	19,037	5.45–5.77
		B	19,006	5.3–5.8
β-lactoglobulin	2–4	A	18,363	5.13
		B	18,277	5.13
α-lactalbumin	0.6–1.7	B	14,178	4.2–4.5
Serum albumin	0.4	A	66,399	4.7–4.9
Immunoglobulin G1	0.3–0.6	…	161,000	5.5–6.8
Immunoglobulin G2	0.05	…	150,000	7.5–8.3
Immunoglobulin A^7	0.01	…	385,000–417,000	
Immunoglobulin M	0.09	…	1,000,000	…
Secretory component	0.02–0.1	…	63,750	…
Lactoferrin	0.02–0.1	…	76,110	8.81

*Calculated as formula weight (3 decimal places) from composition. All acidic groups are protonated, all basic groups are not protonated. Where known, the major disulfide linkages are taken into account; κ-casein has no disulfides, but its N-terminal pyroglutamic is included. Immunoglobulins represent a range.

A comparison of the primary sequences of bovine and caprine milk proteins

There is a substantial degree of homology in the primary sequences of bovine and caprine proteins (table 4). Differences in the primary sequences can however cause the proteins to behave differently and perhaps alter how the proteins are digested.

Due to genetic polymorphism, caprine milk from different breeds (or individuals) may have variable amounts of the same protein and the primary sequences may differ. Genetic variants are caused by point mutations, insertions, deletions and differential splicing patterns of the DNA sequence in addition to post translational modifications (glycosylation/phosphorylations etc). This can alter the transcription rate of the genes and cause differences in the transcribed primary sequences of the proteins. Different primary structure of the same protein can result in molecules that have different shape or size and the hydrophopic/hydrophilic properties of the molecule can be modified [98].

Table 4. Homology between the major proteins in bovine and caprine milk. Abbreviations: κ-casein (κ-CN), α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), β-lactoglobulin (β-LG), α-lactalbumin (α-LA) and amino acid residues (aa) (Data extracted from www.uniprot.org and http://blast.ncbi.nlm.nih.gov/Blast.cgi).

	κ-CN		**α_{s1}-CN**		**α_{s2}-CN**		**β-CN**		**β-LG**		**α-LA**	
	Bovine	Caprine	Bovine	Caprine	Bovine	Caprine	Bovine	Caprine	Bovine	Caprine	Bovine	Caprine
UniProt identifier	P02668	P02670	P02662	P18626	P02663	P33049	P02666	P33048	P02754	P02756	P00711	P00712
Sequence length (aa)	190	192	214	214	222	223	224	222	178	180	142	142
Genetic variant	A	A	B	B	A	A	A^2		B		A	
Signal peptide (aa)	21	21	15	15	15	15	15	15	16	18	19	19
Molecular mass (Da)	21,269	21,441	24,529	24,290	26,019	26,389	25,107	24,865	19,883	19,976	16,247	16,255
Homology	**163/192 = 84%**		**188/214 = 88%**		**197/223 = 88%**		**204/222 = 92%**		**170/180 = 94%**		**135/142 = 95%**	

Caseins

The caseins are a group of phosphoproteins composed of α_{s1}-, α_{s2}-, β- and κ-casein [99] characterised by a high content of proline and a low content of cysteinee/cystine [100]. The high proline content results in very low levels of α-helix or β-sheet and the caseins can therefore be called intrinsically unstructured proteins [93, 101]. All caseins have hydrophilic and hydrophobic areas due to clusters of polar and apolar amino acids.

α_{s1}-casein (α_{s1}-CN) is the primary casein in bovine milk. It has 199 amino acids of which 8 or 9 are phosphorylated and has no cysteinee/cystine. Seven bovine α_{s1}-casein genetic variants have been identified (variants A, B, C, D, E, F, G and H) [93, 97].

α_{s2}-casein (α_{s2}-CN)is the most hydrophilic of the caseins with three clusters of anionic groups composed of phosphoseryl (10-13 phosphate groups per molecule) and glutamyl residues. The protein is composed of 207 amino acids with a high content of lysine and two cysteines per molecule. Four genetic variants for bovine α_{s2}-casein have been identified (variants A, B, C and D) [93, 97].

β-casein (β-CN) has 209 amino acids and is the most hydrophobic of the caseins. It contains no cysteine/cystine and has only 4-5 phosphate groups per molecule. Twelve genetic variants of bovine β-casein have been reported (variants A^1, A^2, A^3, B, C, D, E, F, G, H^1, H^2 and I) [93, 97]. Proteose peptones and γ-caseins are fragments of β-casein cleaved by plasmin in milk [102].

κ-casein (κ-CN) consists of 169 amino acid residues and 11 bovine genetic variants have been identified (variant A, B, C, E, F^1, F^2, G^1, G^2, H, I and J). The C-terminal region is strongly hydrophilic since it lacks aromatic residues, has few apolar residues, and in some cases a high content of sugars, whereas the N-terminal is strongly hydrophobic. κ-casein has 1-3 phosphate groups per molecule and two cysteines. Chymosin (rennin) in rennet cleaves κ-casein at Phe 105 and Met 106, producing para-κ-casein (residues 1-105) and glycomacropeptide (residues 106-169) [93, 97].

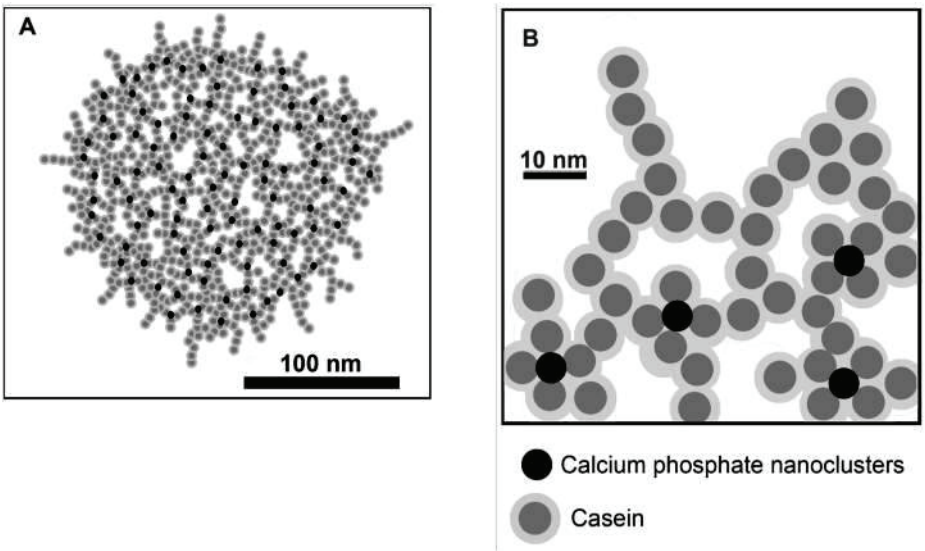


Figure 10. Schematic diagram of an interlocking lattice model of the casein micelle with aggregates of casein-calcium phosphate throughout the supramolecule and chains of proteins extending between them. Cross-sectional scaled views of (A) the complete supramolecule, and (B) a portion of the supramolecule periphery. Calcium phosphate nanoclusters are shown with a diameter of 4.8 nm and approximately 18 nm apart, and caseins are shown with a hydrodynamic diameter of 8 nm (from McMahon & Oommen, 2008 [101]).

The clusters of serine phosphates in α_{s1}-, α_{s2}-, and β-casein make these proteins sensitive to calcium and they would precipitate in milk without the stabilisation offered by κ-casein [93, 97]. In milk, the caseins are therefore arranged in colloidal particles, 50-600 nm in diameter, referred to as micelles [103]. The structure of the casein micelles have been debated for years and a recent model by McMahon and Oommen [101] called the interlocking lattice model is depicted in figure 10. The interior of the micelles consists of α_{s1}-, α_{s2}- and β-caseins. The caseins have phosphoserine domains that bind to calcium phosphate nanoclusters and are also connected through calcium bridges and hydrophobic interactions [101, 104]. On the surface of the micelles κ-caseins are arranged in clusters with the hydrophilic C-terminal part projecting into the watery phase [101, 105]. The κ-caseins provide a net negative charge on each micelle resulting in repulsion between micelles [93].

Micelles serve as the major nutritional source of amino acids, calcium and phosphate to meet the growth and energy requirements of mammalian neonates [101].

Whey proteins

Proteins dissolved in the milk serum are called whey proteins. Whey proteins are composed of β-lactoglobulin, α-lactalbumin, serum albumin, various immunoglobulins, lactoferrin and fragments of β-casein produced by plasmin called proteose-peptones and γ-caseins. If the whey is derived from rennet coagulated milk, it also contains glycomacropeptide, the C-terminal part of κ-casein which is removed by chymosin [93]. All the whey proteins are globular proteins.

β-lactoglobulin (β-LG) accounts for about 50% of the total whey protein content and is therefore the most abundant whey protein in ruminant milk (table 3). The protein is, however, not found in human milk [(94)] and its biological function is still not clear. The 18 kDa protein is synthesised in the mammary gland, is composed of 162 amino acids and has 2 moles of cystine and 1 mole of cysteine per monomer (figure 11) [(93)]. The disulfide bonds in the native protein are between Cys 66 and Cys 160 and between Cys 106 and Cys 119 with Cys 121 as a free thiol. Eleven genetic variants (A, B, C, Dr, E, F, G, H, I, J and W) have been reported in bovine milk and the A and B variants occur in most bovine breeds [(97)]. Figure 10 shows how the compact globular structure of the protein is made up of an 8-stranded antiparallel β-barrel with one major α-helix and a ninth β-strand on the outside [(106, 107)]. Between pH 5.5 and 7.5 all bovine β-LG variants form dimers, but not mixed dimer (pH of milk is approximately 6.5). Below pH 3.5 and above pH 7.5 it dissociated to monomers and between pH 3.5 and 5.5 it forms octamers [(104, 108)].

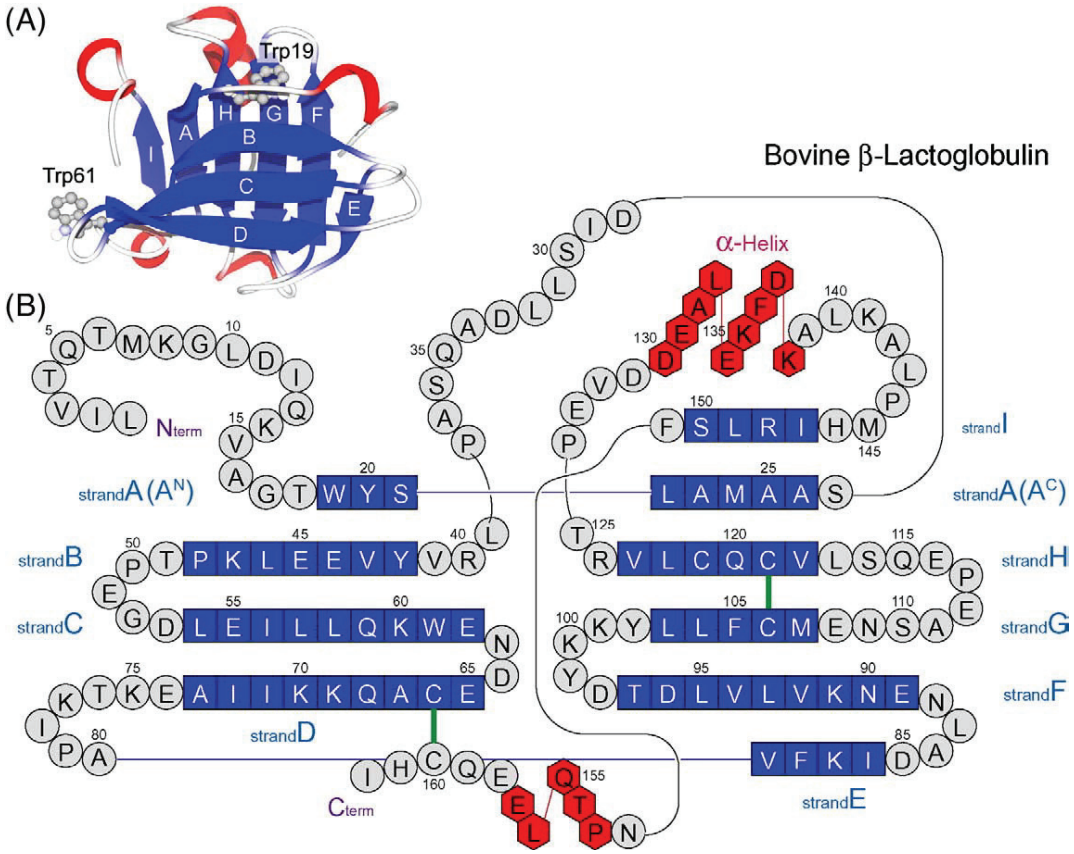


Figure 11. The 3D structure and amino acid sequence of bovine β-lactoglobulin (β-LG). (A) A monomer of β-LG. The β-strands are labeled and tryptophan (Trp) residues are represented as balls and sticks. (B) A schematic representation of the amino acids of the β-LG sequence. Residues making up the α-helix, β-sheet, and loop are represented by hexagons in red, squares in blue, and circles in grey, respectively. Green lines indicate the positions of disulfide bonds. It is seen that β-LG has two β-sheets; The B–D strands and N-terminal half of the A strand (denoted A^N) consist of one sheet and the second sheet is made up of the E–H strands and C-terminal half of the A strand (denoted A^C) (from Sakurai *et al*., 2009 [(106)]).

The protein belongs to the lipocalin family [109], a family of many ligand binding proteins. β-LG has been shown to bind many hydrophobic molecules such as vitamin D, lipids, folic acid, retinol and fatty acids [110-113]. Since β-LG exhibits high stability at low pH, it could therefore be important in the transfer of these hydrophobic ligands to the small intestine [114]. There is, however, disagreement as to the biological importance of this since some species such as humans and horses do not produce β-LG to support the growth of its offspring. Kontopidis *et al*. [115] have proposed that the original biological role could have been related to maternal physiology, but that this has developed to a more nutritional role for some species.

α-lactalbumin (α-LA) is also synthesised in the mammary gland and represents 20% of the total whey protein content. Three genetic variants (A, B and C) of this 14 kDa protein have been identified. It contains 123 amino acids of which essential amino acids (Trp, Phe, Tyr, Leu, Ile, Thr, Met, Cys, Lys and Val) account for about 63% [97]. Four intramolecular disulphide bonds stabilise its globular structure, a structure that shows 63% similarity to that of bovine lysozyme [93, 97].
α-LA is essential for the production of lactose. The protein interacts with β-1,4-galactosyltransferase to form the lactose synthase complex [116]. In this complex, α-LA seems to hold the glucose molecule and thereby inhibits the binding of other substrates such as N-acetyl-glucosamine to β-1,4-galactosyltransferase [117]. Glucose and UDP-galactose are thereby allowed to attach and form lactose. Lactose is the principal molecule in milk affecting the osmotic pressure.
In addition, α-LA has been shown to bind metal ions such as calcium [118] and zinc [119] in a pocket formed by four Asp residues. This pocket is highly conserved in all α-LAs and lysozymes [93]. At pH below 5 the Asp residues become protonated and lose their ability to bind Ca^{2+}.

Serum albumin (SA) in milk originates from blood serum following passive leakage into the mammary gland. This globular protein of 66 kDa is composed of 583 amino acid residues and has 17 disulphide bonds [97, 120]. It represents approximately 8% of total whey proteins and sequence homologies are reportedly high among those mammals studied [97].
SA in blood can bind lipids such as free fatty acids and also flavour compounds [114]. Bovine SA has also been shown to inhibit the growt of human breast cancer cells *in vitro* [121].

Immunoglobulins (Igs) in milk are tranferred from blood to protect the mammary gland from pathogens and to induce passive immunity for the calf since they are too large to pass through the placenta of cows [122]. There are five classes of Igs, but only IgG, IgA and IgM are present in milk. Their concentration and composition vary widely with the highest content found in colostrum, whereas the concentration in late lactation milk is rather low [123]. All the Igs have similar basic structure and are produced by B lymphocytes. IgG, the major Ig class in milk, is composed of two identical heavy chains

(55-76 kDa) and two identical light chains (22.5-27.3 kDa). Disulfide bonds link the two heavy chains together and each light chain to one heavy chain to make two identical binding sites for antigens. IgA is composed of two such units connected through a secretory component and a junction chain. IgM is composed of five four-chain units connected by disulphide bonds and a junction chain [97, 124].
The Igs bind antigens and act as links to other parts of the immune system. Their main functions include: activation of complement-mediated bacteriolysis, opsonisation, prevention of adhesion of pathogenic microorganisms to the mucosal epithelial cells, neutralisation of viruses and toxins and agglutination of bacteria [124].

Lactoferrin (LF) is an iron-binding glycoprotein produced in the mammary gland as well as by other epithelial cells and leucocytes. The protein can therefore be found in mucosal secretions such as tears, saliva, gastrointestinal fluids and urine in addition to milk [125, 126]. The molecular weight of LF differs according to the glycosylation level, but is generally approximately 80 kDa. It consists of a single polypeptide chain consisting of 689 amino acids with five potential glycosylation sites. Lactoferrin is folded into two globular lobes (the N- and C-lobes) stabilised by 17 disulphide bridges. Each lobe is able to bind one ferric ion (Fe^{3+}) together with one carbonate ion and can only be released below pH 3.5 [97, 127, 128].
By binding iron strongly LF is able to protect against potentially harmfull microorganisms that require iron to sustain their growth (bacteriostatic effect). In addition, the cleavage of LF by commercial pepsin has resulted in the production of a 1,545 Da N-terminal peptide called lactoferricin (LFcin, f17-41) [129]. The net positive charge on this peptide enables it to interact with negatively charged molecules on some microorganisms, such as lipopolysaccharide on gram negative bacterial membranes, resulting in lysis of the bacterial cell [126, 130]. Even though LFcin has been detected in the human stomach after ingestion of LF in a relatively high concentration (10 mg/ml) [131] it has not been detected after *in vitro* digestion of LF using human gastric juice at our laboratory (Furlund *et al*. manuscript submitted). An *in vivo* study in adult humans has furthermore demonstrated how a substantial amount of LF (> 60%) may survive gastric digestion and enter the duodenum in an intact form [132].

In addition, milk contains many enzymes of which **lactoperoxidase (LP)** is of great importance as an antibacterial agent. The antibacterial effect is not due to LP alone. LP is involved in the formation of antibacterial oxidation products of thiocyanate only when combined with hydrogen peroxide and thiocyanate [93].

1.3.3 Goat milk

The majority of the milk consumed is of bovine origin, however, milk from other species such as buffalo, goat, donkey and sheep is also frequently used. Caprine milk production increased on a world basis from 1980 to 2001 to approximately 2% of the world's annual milk supply [133]. According to Haenlein, caprine milk has a three-fold

significance in human nutrition: [1)]feeding people in the rural areas of the developing world, [2)]possibly treating people suffering from cow milk allergies and [3)]satisfying the gastronomic needs of consumers [(133)]. For children allergic to bovine milk proteins, caprine milk has been suggested as a good alternative. It has however, been shown that there is a substantial cross-reactivity between caprine and bovine milk proteins [(134)] and that caprine milk should therefore not be used as a bovine milk substitute [(135)].

The composition of milk varies with specie, breed, age, diet, health and stage of lactation [(93)]. Caprine and bovine milks have a similar basic composition. Cow's milk has an average composition of 3.6% fat, 3.3% protein and 4.6% lactose, whereas milk from goat has 3.8% fat, 3.5% protein and 4.1% lactose [(136)].

Goat milk caseins and genetic polymorphism

The composition of caseins in goat milk is strongly influenced by the genetic polymorphism at the casein loci [(137)]. The total concentration of casein in goat milk ranges from 20 to 27 g/l while bovine milk has a total casein concentration of 27 g/l [(138, 139)]. This is primarily due to the high degree of genetic polymorphism of the goat α_{s1}-casein gene causing variable synthesis of the protein, although genetic variants causing variable amounts of the other caseins have also been reported [(98)]. In goats, at least 18 genetic variants of α_{s1}-casein have been reported [(140, 141)] compared to 8 bovine α_{s1}-casein alleles [(97)]. Four different expression levels have been found (null, low, intermediate and high amount) resulting in concentration of α_{s1}-casein ranging from 0 to 4.2 g/l per allele in goat milk [(96)]. A particularly high frequency (>70%) of the indigenous Norwegian goat breed are homozygous for the "null" allele referred to as allele D, which is related to no synthesis of α_{s1}-casein [(141, 142)]. Less or no α_{s1}-casein in milk leads to less total protein and casein concentrations, larger mean casein micelle sizes and a downregulation of lipid synthesis compared to milk with high α_{s1}-casein content [(140)]. In addition, low α_{s1}-casein concentration strongly affects the renneting properties during cheese manufacturing causing weaker gel strength and lower cheese yield [(141)].

In caprine milk, β-casein is the main casein, while α_{s1}-casein is the main casein in bovine milk [(143)]. There is also more α_{s2}-casein and κ-casein in caprine milk compared to bovine milk [(136, 138, 143)].

The casein micelles in goat milk are larger than the cow milk casein micelles (220 and 184 nm on average respectively) [(94)] and contain more inorganic phosphorus and calcium [(144)]. In addition, β-casein is more soluble especially upon cooling than the bovine homolog [(144, 145)] resulting in a larger amount of this protein in the caprine whey.

The content of β-LG and α-LA in goat and cow milks does not differ to the same extent as the caseins. The concentration of α-LA is approximately the same, whereas there is a slightly higher amount of β-LG in bovine compared to caprine milks [(138, 139, 144, 146)].

Table 5. Comparison between the average protein composition in cow and goat milk (mean g/l) (from Martin & Grosclaude, 1993, Farrell *et al*., 2004, Miranda *et al*., 2004 and Moatsuo *et al*., 2008 [96, 97, 138, 139]).

Protein	Goat	Cow
Casein		
αs1-casein	0-8.4*	13.5
αs2-casein	4.2	3.7
β-casein	11.0	10.0
κ-casein	4.6	3.5
Whey proteins		
β-lactoglobulin	2.1	3.3
α-lactalbumin	1.2	1.2
Lactoferrin	0.1	0.1
Serum albumin	0.4	0.4
Immunoglobulins	0.5	0.7
Lysozyme	Traces	Traces

1.4 IMMUNOMODULATING MILK PEPTIDES

1.4.1 Digestion of milk proteins

The primary role of most milk proteins is considered to be a nutrient for the growing offspring. Milk proteins of both cow and goat origin are ranked as high-quality proteins due to their high digestibility and elevated content of essential amino acids [136, 147]. Essential amino acids, and especially leucine [148] is considered primarily responsible for the amino acid-induced muscle protein anabolism in elderly [149], as well as young persons [150]. Whey proteins are more effective than casein at promoting postprandial muscle protein anabolism due to a particularly rich content of leucine, as well as a faster digestion and absorption kinetics [151]. This also makes whey proteins popular products by athletes wishing to promote muscle growth in conjunction with correct training [152]. Caseins on the other hand, have been shown to stimulate pancreatic secretion more efficiently than β-LG [153]. Gastrointestinal hormones controlling the secretion of gastric and pancreatic juice as well as bile are stimulated by dietary proteins [154]. Peptides derived from caseins are suggested to be responsible for part of this stimulation and has been shown to decrease the intestinal transit time compared to whey proteins [155-158].

Fast versus slow dietary proteins

Milk proteins have high digestibility with most of the milk protein nitrogen being absorbed in the duodenum and jejunum. The absorption rate of nitrogen from milk is mainly controlled by the rate of gastric emptying [159, 160].

During digestion, the solid part of a meal is emptied from the stomach more slowly than the liquid part. When milk is ingested the caseins will coagulate in the acid stomach environment at the isoelectric point (pI 4.6) causing them to lose their net charge. Whey proteins on the other hand, remain in solution and will therefore rapidly enter the intestine, mostly as intact proteins, where extensive proteolysis is taking place [153, 161]. At the same time, the pepsins in the stomach perform limited proteolysis on the caseins while peptides of various sizes are slowly emptied from the stomach. Due to the different time of reaching the intestine for complete proteolysis, the amino acids of the whey proteins will be released and absorbed faster, however, more distally compared to the amino acids of the caseins [153]. In comparison, the gradual emptying of partially digested caseins from the stomach observed *in vivo* by adults ensures a prolonged release and absorption of amino acids in the upper part of the intestine. These differences in the speed of absorption have led to the concept of dividing milk proteins into "slow" (casein) and "fast" (whey proteins) metabolized dietary proteins [162]. This concept is, however, debated. An *in vivo* study where human milk was ingested by infants showed that after 1 hour several proteins were resistant to the gastric proteases [21]. The resistant proteins include LF, α-LA and β-CN. The same study also demonstrated that bovine α-LA and β-LG were resistant to digestion with human gastric juice while bovine GMP was rapidly digested. Intact caseins digested *in vitro* using human gastric and duodenal juices from adults have also been shown to be rapidly digested, much faster than for the whey proteins [94]. It has also been shown that hydrolysed casein behaves similar to "fast" proteins with a rapid digestion and absorption *in vivo*, probably because it then does not clot in the stomach [163]. The concept of "slow" versus "fast" protein digestion should also be discussed on the basis of its definition.

Peptic digestion of β-lactoglobulin

Peptic cleavage of β-LG is known to be rather difficult since the preferential cleavage sites of pepsin (hydrophobic amino acids) are situated in the core of the proteins tertiary structure [164-166]. β-LG in ovine milk is hydrolysed faster by pepsin compared to bovine milk β-LG [166]. Five amino acid residues are replaced in the ovine β-LG and four of these replacements are similar for caprine β-LG (www.expasy.org). It was speculated that the increased susceptibility to pepsin was caused by the amino acid replacements leading to an increased surface hydrophobicity and possibly changes in the tertiary structure. Previous research has also indicated that β-LG in caprine milk was digested more rapidly than its bovine counterpart by human gastric and duodenal juices [167]. β-LG is less resistant to hydrolysis by trypsin and chymotrypsin, but the proteolysis is slow [108, 168]. It has also been shown that some intact β-LG or large peptides of this protein can cross the epithelial barrier as trace amount are found in human milk by

mothers consuming bovine milk (β-LG is not present in human milk) [169]. The absorption of partly intact β-LG by human and rabbit intestinal explants has also been observed [170].

1.4.2 Bioactive milk peptides

The low or reduced content of α_{s1}-casein in Norwegian goat milk results in poor renneting properties and therefore reduced cheese yield. Investigations into the alternative use of caprine milk proteins have led to a search for hidden bioactive peptides within these protein sequences.

Bioactive peptides are defined as protein fragments that beside their nutritional value induce desirable effects on body functions or conditions that could eventually influence human health [88, 171]. The peptides are released upon enzymatic cleavage of proteins by digestive enzymes or by bacteria during fermentation [90]. Usually, the peptides contain 3 to 20 amino acid residues and many peptides have multifunctional properties [172].

Bioactive peptides produced from bovine milk proteins *in vitro* using commercial enzymes have been a major research focus for many years and several reviews describing their production and possible physiological effects have been written [88, 89, 91, 173, 174]. Both caseins [92] and whey proteins [175-178] are sources of bioactive peptides. Owing to the high sequence similarities between bovine and caprine proteins many of the identified bioactive bovine milk peptides could also be produced through digestion of caprine milk proteins. However, as described for β-LG [166], small changes in the amino acid sequences of proteins can change the structure and the exposure of amino acids required for the different digestive proteases. This could result in a slightly altered pattern of peptides released.

Milk of different species varies in its protein composition, in the relative amounts as well as in the amino acid composition of each protein. This leads to variable content of peptides after enzymatic proteolysis. Conditions during proteolysis, such as temperature and what type and amount of enzymes used, also greatly affects the formation of peptides and thereby also which biological activities will be present in the final product. Depending on their amino acid sequence, bioactive peptides could, through consumption, affect the main body systems – the cardiovascular, digestive, immune- and nervous systems [88]. Milk peptides have been found to have opiate, antithrombotic, antihypertensive, antioxidative, immunomodulating, antimicrobial and mineral carrying properties [174, 179]. At the luminal side of the intestine the peptides could interact with enzymes or epithelial cell surface receptors. Alternatively some bioactive peptides could induce their effects after being absorbed by epithelial cells and possibly transported to other parts of the body [91, 173, 180].

The many biological activities possessed, in addition to the great quantity of milk, and the rather simple degradation process needed for peptide production makes milk and milk peptides very popular as ingredients in functional foods and nutraceuticals. In Finland, Sweden, the Netherlands, Japan and USA commercial dairy products supplemented with bioactive milk protein derived peptides already exist and clinical human studies have been performed to document the health effects of these products [91].

1.4.3 Immunomodulating whey peptides

Through enzymatic hydrolysis of milk proteins a number of peptides that could potentially affect cells of the immune system are released. It has been suggested that immunomodulatory milk peptides may be part of regulating the development of the immune system in infants and to ease allergic reactions in atopic humans [90]. The immunomodulating effects caused by whey proteins, their hydrolysates and individual milk protein-derived peptides are diverse. While some milk protein components seem to stimulate immune functions, other protein components in the same milk sample may exhibit immunosuppressive effects [180].

Research has often compared the effect of intact milk proteins (single or in mixtures) with their enzymatic hydrolysates and data are lacking on the characterisation of individual immunomodulating peptides [177]. Loss of immunomodulating effects have also been shown after enzymatic digestion of milk proteins, particularly after a high degree of proteolysis [181].

In order to induce significant effects on the adult immune system it will likely be necessary to enzymatically produce and concentrate these peptides on a commercial scale as the amount produced during *in vivo* digestion is probably too low [177].

Effects on the adaptive immune response

Bioactive peptides could affect the adaptive immune system by modulating the activation and proliferation of lymphocytes, the production of antibodies and the release of various cytokines.

Recently Saint-Sauveur *et al*. [182] showed that a whey protein isolate (WPI) and its digest produced by trypsin and chymotrypsin stimulated the proliferation of resting murine splenocytes in a concentration-dependent manner. It was further shown that peptide fractions (<10 kDa) of the enzymatic digest increased the proliferation of both resting and concanavalin A (ConA) stimulated splenocytes. The WPI on the other hand suppressed the the ConA stimulated proliferation. While the enzymatic digest slightly stimulated the secretion of IL-2 and IFN-γ by the resting splenocytes, the peptide

fractions significantly enhanced the secretion of these cytokines indicating a shift towards the Th1 response [182].

Purified β-LG stimulates the *in vitro* proliferation of murine spleen cells [183, 184], there is however some inconsistency as to the effect of β-LG peptides on these cells. Mahmud *et al*. [184] showed that digestion of β-LG with pepsin, trypsin, chymotrypsin or pancreatin induced a stronger mitogenic effect compared to the intact protein. Wong *et al*. [183] on the other hand, reported a reduced effect of tryptic β-LG peptides. β-LG is not present in human milk [138] and is one of the proteins responsible for milk allergies in children [185]. Tryptic peptides from β-LG have, however, been suggested to protect against allergy by inducing oral tolerance and reducing IgE towards β-LG [186].

In bovine cheese whey κ-casein has been digested with chymosin releasing fragment 106-169 called glycomacropeptide (GMP). Both intact and partially digested GMP has been observed in the blood circulation of humans after milk or yoghurt ingestion [187]. This peptide has demonstrated inhibitory effects towards lipopolysaccharide (LPS)- and phytohemagglutinin (PHA)-induced proliferation of murine spleen lymphocytes *in vitro* [188-190]. This indicates that GMP is able to suppress both B- and T-cell proliferation since LPS mainly stimulates B cells while PHA stimulates T cells [189]. GMP was shown to adhere directly to the $CD4^+$ T-cell surfaces and to suppress the expression of the IL-2 receptor [191]. In addition, a modified whey protein concentrate rich in GMP has been shown to suppress mitogen-induced proliferation of both T and B cells [181]. This WPC also suppressed the splenocytes secretion of IL-4 and IFN-γ. *In vitro* digestion by commercial pepsin and pancreatin however partly abolished these effects. An inhibition of IFN-γ as well as tumor necrosis factor (TNF)-α production by ConA activated murine splenocytes was also observed by Requena *et al*. [192]. They furthermore found that GMP enhanced the expression of forkhead box P3 (Foxp3) and secretion of IL-10 in non-stimulated splenocytes. Others have found that κ-casein digested with pepsin and trypsin stimulated the mitogen-activated proliferation of human lymphocytes [193]. In addition, GMP has been to suppress serum IgG production by mouse lymphocytes [194].

Two synthetic peptides, namely κ-casein fragment 38-39 (Tyr-Gly) and α-LA fragment 18-20 (Tyr-Gly-Gly) have been shown to enhance the *in vitro* proliferation as well as protein synthesis in ConA stimulated human peripheral blood lymphocytes [195]. These peptides could reach lymphocytes in the nearby lymphoid tissues since they theroretically are small enough to pass through the intestine [180].

β-casomorphins are fragments of bovine β-casein that have opiate-like properties and have been shown to modulate the immune system [180]. These peptides have also been found in the intestinal juices of people that had ingested bovine milk [196] and it is therefore assumed that they are formed during *in vivo* digestion of bovine milk. β-casomorphin-7 (f60-66) and β-casokinin-10 (f193-202) have been found to suppress *in vitro* lymphocyte proliferation at lower concentrations while higher concentrations increased their proliferation [195].

Miyauchi *et al*. [197] demonstrated that LF digested by commercial pepsin increased the proliferation of murine spleen B cells and enhanced their production of IgM, IgG and IgA. The hydrolysate also enhanced the proliferation and IgA production of Peyer's Patch cells. This effect was, however, abolished following digestion with commercial trypsin. In contrast, intact LF showed an inhibitory effect on murine splenocyte proliferation *in vitro* [192, 197] and reduced the production of TNF-α, IFN-γ and IL-2 [192]. Feeding LF to mice has also been found to stimulate the mucosal immunity as revealed by Debbabi *et al*. [198]. In agreement with Miyauchi *et al*. [197], they showed that ingested LF enhanced the secretions of IgA and IgG in both Peyer's patches and in the spleen. Administration of LFcin to mice, either orally or subcutaneously, has also shown promising results against cancer cells by inhibiting metastasis and tumour growth [199]. An anti-cancer effect of LF consumtion by mice has also been demonstrated by Varadhachary *et al*. [200]. Oral LF enhanced the lytic activity of spleen NK-cells and expanded circulating CD8+ T cells primarily by production of IL-18 in the gut [200]. IL-18 activates T- and NK-cells and increases IFN-γ production in the intestinal epithelium of mice [201]. When added to human polymorphonuclear leukocytes *in vitro*, both intact bovine LF and LFcin have been shown to stimulate the release of neutrophil-activating IL-8 [202]. LFcin has also been hypothesised to reduce the risk of cancer by milk consumption as *in vitro* incubation of LFcin with a human colon cancer cell line (CaCo-2) in physiologically relevant doses (0.2 L/day) has been demonstrated to prolong the S phase of the cell cycle [203]. After a 5 week treatment of these cells with LFcin, cell proliferation was significantly delayed [203].

Effects on the innate immune response

A limited amount of studies have examined the effect of whey protein derived peptides on the cells of the innate immune response. Milk peptides could affect the proliferation of these cells, the phagocytic activity of macrophages (and other phagocytosing cells), as well as their production of cytokines.

The incubation of a murine monocytic cell line with GMP induced the expression of an IL-1 receptor antagonist (IL-1ra) [190]. GMP thereby blocks the action of IL-1 by binding of the IL-1ra to IL-1 receptors avoiding the activation of splenocyte proliferation and thereby an inflammatory response. Li & Mine [204] demonstrated that GMP enhanced the proliferation and phagocytic activity of human macrophage-like cells (U937). Pepsin digestion of GMP increased this immunostimulatory effect. In addition, Requena *et al*. [205] has shown that incubation of human monocytic cells with GMP increased the production of IL-8, TNF-α and IL-1β via the activation of the NF-κB and the MAP kinase pathways [205].

Bovine LFcin has been shown to suppress IL-6 production by a human monocytic cell line in response to *in vitro* LPS stimulation [206]. In addition, LFc promotes the

phagocytic activity of human neutrophils [207] and increases the production of IL-18 and IFN-γ [208].

Another whey protein that influences macrophages is α-LA. This protein increased the production of IL-1b by sheep macrophages [209]. Additionally, a tripeptide from human α-LA (GLF, f51-53) has been shown to bind to specific receptors on human monocytes and macrophages *in vitro* thereby stimulates the adherence and phagocytic activity of these cells [210, 211]. Jaziri *et al*. [211] also reported that human β-casein fragment 54-59 (VEPIPY) stimulates the phagocytic activity of human monocytes by binding to a different receptor.

Bovine β-casein fragments f191-193 and f63-68, have been shown to *in vitro* stimulate the phagocytic avtivity of macrophages [212].

There is an increasing amount of research performed to support the theory that digestion of whey protein can produce immunomodulating peptides. Unfortunatly, the results are sometimes contradictory possibly due to the use of different methodologies, enzyme sources, raw materials and digestion models [177]. In addition, the *in vivo* production of these peptides or survival of consumed commercially produced peptides through the gastrointestinal tract remains to be prooved. It is also of importance to investigate by which mechanisms the immunomodulating peptides act. This includes a study of the transport of such peptides across the epithelial cell layer to ensure that the peptides reach the gut associated lymphoid tissue where they can excert their actions.

2. AIMS OF THE STUDY

An *in vitro* static digestion model using human gastric and duodenal juices to digest milk proteins had previously been developed in our laboratory [167]. To follow up these studies, this project aimed at identifying potentially bioactive peptide sequences produces after *in vitro* digestion of caprine whey proteins using human gastric and duodenal juices.

The study was divided into four subgoals:

a) *To characterise the gastric and duodenal juices used during in vitro digestion with regard to individual differences and stability of enzymes during frozen storage.*

b) *To compare digestion of whey proteins using either human gastric and duodenal juices or commercial porcine digestive enzymes.*

c) *To identify peptides produced after in vitro digestion of whey proteins.*

d) *To screen for effects of identified whey peptides on in vitro cell responses – on cultures of mixed cell types such as PBMCs and on individual cell types such as T cells and dendritic cells.*

3. SUMMARY OF PAPERS

Paper I

Human gastrointestinal juices intended for use in *in vitro* digestion models (2011)

In this paper a method used for the simultaneous collection of human gastric and duodenal juices was described. Individual gastric (HGJ) and duodenal (HDJ) juices fom 20 volunteers were collected. HGJ samples were analysed for their pepsin activity as well as their content of protein, while total proteolytic, amylase and lipase activities as well as bile acids, protein and bilirubin concentration were measured in the individual HDJ samples. Large individual differences were detected in the total volumes secreted as well as in the enzyme activities and duodenal bile acid concentrations.
Batches of HGJ and HDJ were made to facilitate the use of the same digesitve juices for different digestion studies. The activities of pepsin (HGJ) and lipase (HDJ) were, however, gradually lost during frozen storage, thus it was recommended that human digestive juices intended for use in *in vitro* digestion studies should be used within two months after collection.

Paper II

Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes (2010)

In this paper, whey proteins were *in vitro* digested in two steps simulating gastric and duodenal digestion. Either human gastric and duodenal juices or commercial porcine digestive enzymes (pepsin A and pancreatic enzymes) were used at the same enzyme activities. In addition, the gastric step was performed at pH 2, 4 or 6 to simulate normal variations in pH during digestion. Increasing the gastric pH reduced the degradation of the proteins, and seemed also to delay the duodenal digestion. Purified commercial pepsin A degraded whey proteins more efficiently than human gastric juice at all gastric pH values. Nevertheless, after the duodenal digestion only β-LG and α-LA were significantly more degraded by the commercial enzymes compared to the human gastroduodenal juices. In addition, we observed that digestion of proteins by human digestive juices gave rise to different peptides when compared to digestion with commercial porcine enzymes. Consequently the use of human digestive juices might be preferred when simulating human digestion.

Paper III

Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juices (2011)

In this study, LC-MS/MS (LC-Q-TOF) was used to identify peptides in the range of 800-4500 Da in a whey protein hydrolysate digested with human gastroduodenal juices. The hydrolysates were fractionated to improve the separation of peptides prior to mass spectrometric analysis. More than 100 whey peptides were identified by LC-Q-TOF and 22 of these peptides originating from β-LG, β-CN and κ-CN were synthesised. No peptide corresponded to the antibacterial caprine lactoferricin f(14–42) or lactoferrampin C f(268–284) was identified. A consensus sequence LTPVPELK was also identified by multiple sequence alignment of nineteen peptides with proline-rich sequences and neighbouring leucines. Both the hydrolysate and single peptides were screened for antibacterial effects. The hydrolysate generated by human gastric and duodenal juices demonstrated strong activity against *Escherichia coli* K12, *Bacillus cereus* RT INF01 and *Listeria monocytogenes*, while no effect against growth of *Lactobacillus rhamnosus* GG was detected. The hydrolysate fraction of the highest molecular weight (above 8 kDa) was stronger than the low molecular weight fractions. The peptides showed less antibacterial effect compared to the hydrolysates.

Paper IV

Effect of milk proteins and their hydrolysates on *in vitro* immune responses (2008)

This paper describes a screening of different milk protein samples for *in vitro* immunomodulating properties on human peripheral blood mononuclear cells (PBMC). Both intact proteins and their hydrolysates generated by *in vitro* digestion with either human gastroduodenal juices or commercial porcine digestive enzymes were studied. Particularly the whey protein concentrates (caprine and bovine) demonstrated a dose-dependent inhibition of proliferation of mitogen-activated PBMCs. The digested whey proteins appeared more inhibitory than the intact proteins. The reduced proliferation seemed to be caused neither by a toxic effect nor by induction of apoptosis.
Furthermore, incubation of whey protein samples with T cells restimulated with anti-CD3 coated beads resulted in an even more profound suppressive effect on proliferation. We suggested that intact or hydrolysed components in the whey protein samples affect the production of activation signals, thereby inhibiting lymphocyte proliferation.

Paper V

Immune cell cytokine profiles induced by different milk derived peptides (manuscript)

In this paper peptides from a whey protein concentrate hydrolysed by human gastroduodenal juices were identified by LC-MS/MS. Synthetic peptides from β-LG, β-CN and κ-CN as well as intact hydrolysates were screened for their immunomodulating effects on purified T cells (adaptive immunity) and dendritic cells (DCs; innate immunity). Digested whey protein concentrate reduced the proliferation of T cells restimulated by anti-CD3 coated beads by about 90% while the most inhibitory fraction of this hydrolysate (Mw <5 kDa) displayed a 50% reduction in T-cell proliferation. The synthetic peptides displayed variable effects on T cell proliferation with 30 % reduction in proliferation at the most. The WPCG hydrolysate fractions and peptides were screened for their effects on T-cell secretion of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ and MCP-1. Unique effects on cytokine release were observed for many of the peptides. In general, all the samples tested increased the secretion of IL-1β and IL-12, while the secretion of IL-4 and IL-5 was reduced by many of the samples. The release of IL-2, IL-6, IL-8, IL-10, MCP-1 and IFN-γ was increased by most of the fractions and peptides tested. Two β-CN peptides (f81-91 and f 144-151) increased the secretion of IL-6 when incubated with DCs. The DC secretion of IL-8 was also increased by most of the tested samples, whereas the levels of IL-10, IL-12 and IFN-γ were unaltered.

4. KEY RESULTS AND GENERAL DISCUSSION

IN VITRO GASTROINTESTINAL DIGESTION MODELS

Much effort has during the last decades been focused on identifying bioactive peptides within the sequences of milk proteins. The release of such peptides has frequently been studied *in vitro* after digestion with proteolytic starter cultures (in cheese and yoghurt) and after *in vitro* digestion using gastrointestinal or microbial enzymes [213-215]. Although many bioactive peptides have been identified, their production and survival in the gastrointestinal tract has rarely been shown. In paper II-V a simple two-step *in vitro* digestion model was used to study the digestion of whey proteins using human gastric and duodenal juices (figure 12). The degradation patterns of the whey proteins were evaluated by 1D gel electrophoresis (SDS-PAGE) and peptides generated were identified by mass spectometry (nano-LC-MS/MS). The identification of peptides depends on the separation methods and the sensitivity of the analytical equipment.

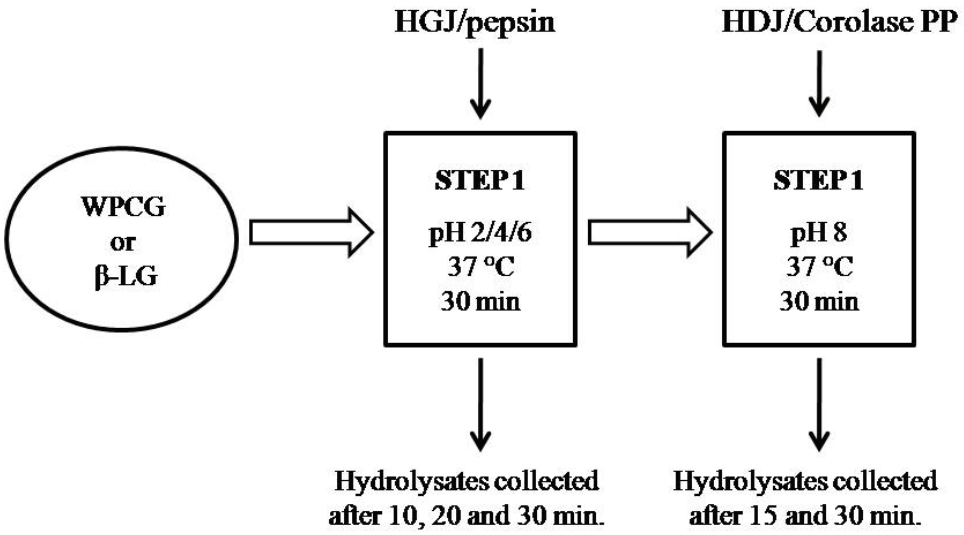


Figure 12. Illustration of the *in vitro* digestion model (paper II-V). Caprine whey protein concentrate (WPCG) or bovine β-lactoglobulin (β-LG) were digested in two consecutive steps simulating human gastric and duodenal digestion. Either human gastric (HGJ) and duodenal (HDJ) juices or commercial porcine pepsin A and pancreatic enzymes (Corolase PP) were used.

Static versus dynamic digestion models

The hydrolysis model used in this study is a simple static model that does not include simulation of movements in the gastrointestinal tract. When studying digestion of milk it is well-known that *in vivo* the reduced pH in the stomach leads to the precipitation of caseins and thereby a delay in the gastric emptying of these proteins [160]. In a static model this is not accounted for as all proteins to be digested are present at all times. Nevertheless, the model may be sufficient when studying the digestion of whey proteins since they remain in solution and are rapidly released from the stomach. Advantages of

the model include its simplicity, easy handling and low cost and it provides rather quick estimates of the degradation patterns of different proteins at chosen pH-values and incubation times.

Dynamic digestion models mimic physical and mechanical processes and temporal changes occuring *in vivo*. Such a model, the dynamic gastric model and small intestinal simulation developed at the Institute of Food Research (Norwich, UK) is validated by comparing *in vitro* and *in vivo* results [43]. It has the advantage of including simulations of peristalic movement in the gastrointestinal tract and pH and enzyme amount are regulated on-line [216]. Using this model could have provided additional information regarding the degradation of whey proteins. Unfortunately, such models are expensive to run and due to their complexity they are not routine equipment.

Human gastroduodenal juices versus commercial digestive enzymes

Since methods measuring enzyme activities are highly reliant on the incubation temperature, pH and the substrate added, the enzyme units used in different publications may not be comparable. In paper II we therefore measured the pepsin activity of commercial porcine pepsin A (Fluka BioChemika) and the total proteolytic activity of Corolase PP (extract from pig pancreatic gland, Röhm) by the same methods used for the corresponding human digestive juices. To study whether the commercial enzymes would digest proteins differently compared to the human digestive juices, whey proteins were hydrolysed by porcine pepsin A and Corolase PP at similar enzymatic activities as for hydrolysis with HGJ and HDJ, respectively (5U gastric enzymes and 16U duodenal enzymes per 10 ml 5% WPCG, Paper II). Many papers describe the use of commercial enzymes to study digestion and report the amount of added enzyme in g or mg instead of units of activity. The activity of different enzyme preparations of one type of enzyme (e.g. pepsin) may vary and storage may decrease the activity. It is therefore important to explain how much enzyme is added in units and to describe the method used to calculate the enzyme activity. This type of standardisation would facilitate comparisons between studies.

The results in paper II indicated that purified porcine pepsin A digested the whey proteins more rapidly compared to the human gastric juice. 90% of human gastric juice is composed of pepsin A and the human and porcine versions of this enzyme have high sequence similarities [13]. Kitabatake and Kinekawa [164] has also demonstrated that similar hydrolytic patterns were generated when β-LG was digested by either human uropepsin (similar substrate specificity as for human gastric pepsin) or porcine pepsin A. Nevertheless, pepsins from different species could exhibit different activities towards the same substrate [16]. Human pepsin A may have a lower affinity for whey proteins compared to the porcine homolog eventhough the amounts added during hydrolysis were based on similar pepsin activities. The reason for this might be that the pepsin activity was calculated after incubation with hemoglobin and the enzymes may have different affinities for this substrate compared to the whey proteins. The reduced

capacity of the gastric juice to hydrolyse whey proteins could also partially be explained by the presence of other isoforms such as gastricsin that may not be as efficient as the pepsin A at digesting whey proteins.

The differences in protein degradation between the human juices and commercial enzymes were not as evident after the duodenal step. While duodenal juice is composed of the collective secretions from the pancreas, the gall bladder and duodenal epithelial cells, Corolase PP is an extract from pancreatic glands. Gass *et al*. [32] has recently shown that the prensence of bile could greatly enhance the digestion of proteins and this could be one reason why the duodenal juice seems so efficient at degrading the whey proteins. In addition, brush border membrane peptidases present in the duodenal juice could have contributed to the degradation.

The enhanced digestion in the gastric step could furthermore have masked differences later on during the digestion. As an example, almost no intact LF remained after digestion with porcine pepsin A at pH 2 and the effect of digestion with Corolase PP on the shorter LF fragments may not be visible on SDS-PAGE.

Enzyme units

Amounts of added HGJ and HDJ in paper III-V were not based on *in vivo* secretion levels but where rather chosen after preliminary trials in the lab [217]. The selected amounts of HGJ and HDJ to be added were those that after 30 minutes incubation with a protein sample resulted in only a partial degradation. Increasing the incubation time from 30 to 90 minutes did not result in more protein degradation as observed by SDS-PAGE according to Almås [217].

During a 2h-collection period large intersubject variations in the total volume of HGJ and HDJ was observed. Total volumes of HGJ varied from 2-355 ml (average of 87 ± 103 ml) and HDJ varied from 10 to 312 ml (average of 158 ± 91 ml). *In vivo*, enzyme secretion is stimulated by the presence of food in the stomach, by hormones and by the mere thought, sight or smell of food [9]. The amounts of the digestive juices added in our studies were in the lower range of the observed volume outputs and are therefore possibly too low compared to the amounts secreted postprandially.

Gastric pH

The pH levels in the human stomach vary both between people and within individuals [9] and in the fasting state it is generally reported to be about 1.0-3.5 in adults [2]. In young children, however, the pH may be much higher. About 1-10 days after birth the neonate's gastric pH is increased to 6-7 and pH 1-2 is not reached until the age of 20-30 months [10]. In addition, a large part of the adult population struggles with gastrooesophageal reflux disease and many are currently on medication such as proton pump inhibitors to releave symptoms [18]. Proton pump inhibitors increase the gastric pH levels to a 24h steady-state of above 4 [18]. Others may have elevated gastric pH due to reduced or absent gastric acid secretion [9]. Ingested food also temporarily raises gastric

pH levels [23, 216] so that there is a suboptimal pH for pepsin activity at the start of the digestion. For this reason we examined how the gastric digestion of whey proteins was affected by pH 2, 4 and 6 representing normal variations in gastric pH levels. As expected the digestion was most efficient at pH 2, with reduced digestion at pH 4 and hardly any degradation of the proteins visible following gastric digestion at pH 6. Duodenal digestion was delayed by increasing gastric pH levels thereby stressing the importance of including a gastric step in *in vitro* digestion models.

Aspirations of human gastrointestinal juices

A continuous amount of research aims to characterise the composition of human gastric and intestinal fluids in both fasted and different fed states in order to make synthetic fluids with simulated composition [23, 51, 53, 56]. Nevertheless, the artificial fluids are far from complete and therefore the use of the actual human digestive juices might be preferred. In addition, the complex combination of enzymes and isoenzymes as well as inhibitors, phospholipids and bile salts in human digestive juices provides a physiological fluid unlike purified enzymes of non-human origin and commercial bile salts dissolved in different artificial gastrointestinal media. Porcine pancreatin, a pancreatic homogenate, contains all the pancreatic enzymes and is often used to simulate duodenal digestion [164, 218], however, this product does neither contain bile acids nor brush border membrane peptidases produced by the epithelial cells. By mimicking human digestion of proteins *in vitro* using human gastric (HGJ) and duodenal (HDJ) juices it seems likely that the situation is more close to that experienced *in vivo* [51]. To perform *in vivo* digestion studies approvals by ethical committees are required. The risk of injury or the degree of discomfort for the volunteer also needs to be considered. Nevetheless, *in vivo* digestion experiments should be performed to validate *in vitro* models to ensure that these are predicting in vivo characteristics [51].

Paper I describes the simultaneous aspiration of HGJ and HDJ (based on Holm *et al*., [219]). This was accomplished by inserting a three-lumen tube designed by Maxter Catheters (Marseille, France) through the nose or mouth of the volunteers. In the fasting state secretion of pancreatic juice is limited following a cyclical pattern closely associated to upper gastrointestinal motility [9]. A long collection period would therefore be required to aspirate a sufficient volume of HDJ during the fasting state. After ingestion of a meal, postprandial pancreatic enzyme secretion is induced within a few minutes. In general, pancreatic enzymes are secreted approximately in parallel. Initially a rapid increase in the enzyme delivery into the duodenum is observed reaching maximal values within the first postprandial hour. The secretion of enzymes is further decreased to a stable rate which last until approximately 3-4 hours postprandially [9]. The concentration of bile is also increased after ingestion of food [220]. In order to stimulate the secretion of pancreatic juice and bile, a stimulatory solution containg saline, valine and phenylalanine was instilled close to the papilla of Vater and the duodenal juice was collected about 10 cm distally. In addition, the juices were intended for future *in vitro* digestion studies so their composition should preferably reflect that of

the so-called fed-state digestive juices. An enhanced secretion of pancreatic juice could be achieved through the use of test meals. However, the presence of added proteins, lipids or carbohydrates in the gastric and duodenal juices would have interfered with their future use. The stimulation of pancreatic secretion by essential amino acids ensured suffient flow of pancreatic juice [221] and bile without adding any contaminating substances. The gastroduodenal juices were therefore neither collected in a completely fasting nor in the fed state.

Individual differences

The *in vitro* digestion of whey proteins performed in paper II-V used HGJ and HDJ from one person only. Due to a large demand it was, however, desirable to collect digestive juices from as many individuals as possible to make batch samples of HGJ and HDJ that could be used for several digestion studies. This would facilitate comparisons of different studies and reduce the possible compositional variations otherwise present.

As stated earlier, the secreted volumes of HGJ and HDJ varied greatly between individuals. The different volumes could partly be explained by leakage of gastric juice through to the duodenum or by reflux of duodenal juice to the stomach. For 10 of the 18 volunteers substantial volumes of digestive juice with a pH<4 and high pepsin activity and low total proteolytic acitivity (pH 8) was collected from the duodenal tube. A substantial variation in individual volumes of duodenal juice was also observed by Moreno *et al*. [222].

The enzyme activities measured in the individual HGJ and HDJ also varied greatly as did the concentration of bile acids in the HDJ and no correlation was found between the measured components. Variations in the composition of human digestive juices in both fasting and fed stages as well as individual differences have also been reported by others [23, 53, 58, 220]. In addition, variability in the pepsin and total duodenal proteolytic activities in gastroduodenal juices aspirated from one individual was also demonstrated. This indicates that using a fixed level of either the human juices or of commercial enzymes and bile acids to perform *in vitro* digestion may be an oversimplification excluding intra- and intersubject physiological differences. Perhaps a more complete picture of the digestive processes would be achieved if *in vitro* digestion was performed with low, intermediate and high levels of added enzymes (human or commercial).

Stability of enzymes during frozen storage

Pooled samples of individual HGJ and HDJ were made to be able to perform several comparable digestion studies using the same juices. However, the stability of the enzymes in these pooled digestive juices is crucial if they are to be used over time. While amylase and total duodenal proteolytic activity was found to be rather stable during frozen storage, the activities of pepsin and lipase were decreased. It has been recommeded that gastric juice should be stored frozen in buffers of pH above 2 or with about 12% glycerol to protect it from autocatalysis [223]. We were, however, not able to

stabilise the pepsin neither by pH adjustment (pH>4) nor by the addition of 13% glycerol.
Lipase has by others been demonstrated to be degraded by chymotrypsin in stored duodenal juice [224, 225]. No protection of the lipase activity was, however, observed when adding inhibitors of chymotrypsin and trypsin in paper I. Addition of such protease inhibitors was anyway out of the question since the duodenal juice was intended for protein digestion. We therefore suggested that the HGJ be used within two months and the HDJ within four months following aspiration to avoid loss in pepsin and lipase activities, respectively.

IDENTIFICATION OF PEPTIDES BY MASS SPECTROMETRY

A major goal of this study was the identification of the peptides generated by the *in vitro* digestion of whey proteins. Caprine whey proteins include β-LG, α-LA, LF, SA, immunoglobulins [138]. In addition, whey produced by rennet coagulation of the caseins contains κ-CN glycomacropeptide. Furthermore plasmin in milk generates many β-CN fragments that are present in whey, especially if the milk has been refridgerated prior to renneting [102]. When caprine whey proteins were digested an array of peptides of various sizes originating from all the precursor proteins were generated. Preliminary studies injecting the complete hydrolysate directly into an electrospray (ESI) quadrupole-time of flight (Q-TOF) mass spectrometer resulted in the identification of only a few peptides. These peptides originated from β-LG, β-CN and κ-CN as these are the most abundant proteins in whey.

To improve the separation of peptides and thereby identify a larger amount of peptides, several fractionation steps were performed (paper II, III and V). The whey protein hydrolysates from both gastric and gastroduodenal digestion in paper III and V were first fractionated using membranes with cut-off at 5 and 8 kDa. The five subfractions were then subjected to size exclusion chromatography and the peptides within each resulting peak were identified by nano-LC-Q-TOF (figure 13). In paper II, the WPCG hydrolysates were only fractionated by size exclusion chromatography before peptide identification by LC MS/MS analysis.

The generation of peptides after *in vitro* digestion is highly reliant on the enzymes used as well as other incubation conditions such as temperature and pH as well as incubation time. In addition, the chosen identification method may influence which peptides are detected. The Q-TOF used to identify the peptide in this study was set to aquire data over a mass:charge ratio of 400-1500 Da detecting peptides with two or three charges.

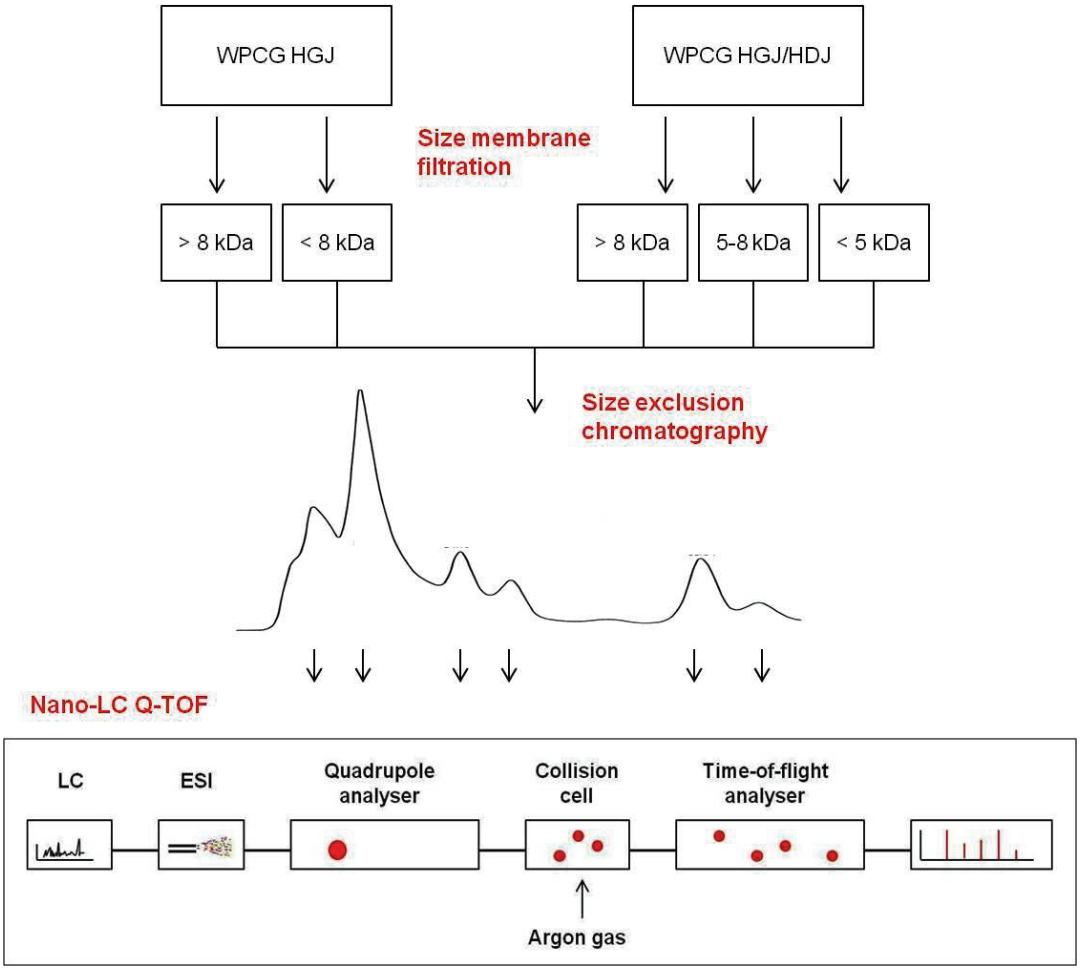


Figure 13. Outline of the fractionation of whey protein hydrolysates (WPCG HGJ and WPCG HGJ/HDJ) before the identification of peptides by nano-LC Q-TOF (paper III and V). The hydrolysates were first fractionated by size membrane filtration using membranes with cut-off at 5 and 8 kDa. Peptides in these fractions were further separated by size exclusion chromatography. Peptides within each peak were identified by MS/MS using nano-LC-Q-TOF. WPCG HGJ – whey protein concentrate digested with human gastric juice, WPCG HGJ/HDJ - whey protein concentrate digested with human gastric and duodenal juice, LC – liquid chromatography, ESI – electrospray ionisation.

Peptides of molecular masses between 800 and 4500 Da could thereby be detected. A mass:charge ratio below 400 Da would give increased background of non-peptide character whereas larger peptides would give poor or missing MS/MS spectra due to reduced fragmentation.

In paper II the amount of β-LG peptides identified in whey digested by either human gastroduodenal juices or commercial pocine enzymes was compared. The β-LG peptide profiles after digestion with porcine enzymes showed a higher amount of peptides covering larger areas of the original protein compared to the digestion with human juices. This indicates that a more extensive degradation was performed by porcine enzymes. Appendix 1 shows the β-LG peptides identified after digestion with human digestive juices or commercial enzymes at gastric pH 2, 4 or 6 (paper II).

As shown in paper III and V a total of 98 peptides were identified after simulated gastric digestion. Most of these peptides were of β-CN origin (44 peptides), however, 12, 46, 9 and 10 peptides were also identified from β-LG, κ-CN, SA and LF. After the duodenal digestion, 109 peptides were identified. The majority of these peptides were of β-LG origin (43 peptides) but 23 and 25 peptides were also identified from κ –CN and β-CN respectively. In addition, 7, 5 and 6 peptides were identified from α-LA, SA and LF, respectively. Thus the prefractionation steps greatly enhanced the identification of peptides. An overview of these peptides is shown in appendix 2.

In paper III and V peptides originating from almost the entire β-LG primary sequence were identified except for regions containing cysteinee groups. In a study where β-LG was *in vitro* digested by porcine and bovine digestive enzymes 12 of the peptides identified were the same as those found in our studies by using human digestive juices [(226)]. We suggested in paper II that the digested β-LG might contain cyclic peptides linked through Cys66-Cys160 and/or Cys106-Cys119/121that were not identified. Both Pequet *et al.* [(227)] and Moreno *et al.* [(226)] identified such peptide fragments linked by two cysteines. Identification of peptides produced by *in vitro* digestion of whey and caseins using porcine pepsin and pancreatic enzymes as well as human brush border membrane proteases was shown by Picariello *et al.* [(228)]. However, only a few peptides originating from β-LG and β-CN were the same as those identified by us.

Survival of milk peptide sequences during *in vivo* digestion

Only a few *in vivo* studies have been performed to evaluate the digestion of milk proteins. It is challenging to get representative samples during the digestion of milk since the aspiration of coagulated proteins is dependent and limited by the tube diameter. When human milk was ingested by infants it was shown that several milk proteins were resistant to the gastric proteases [(21)]. The resistant proteins include LF, α-LA and β-CN. In adult humans after milk or yoghurt ingestion, the appearance of peptides in the stomach, small intestine and blood has been investigated. Many peptides derived from caseins have been detected in the stomach and shorter peptides derived from casein and lactoferrin have been recovered from the small intestine. Both intact κ-CN glycomacropeptide (GMP) and peptides were absorbed and detected in plasma [(187)].

EFFECTS OF WHEY PROTEINS AND PEPTIDES ON *IN VITRO* IMMUNE RESPONSES

In paper IV it was demonstrated that caprine and bovine whey protein concentrates and their digesta reduced the proliferation of mitogen-activated PBMCs in a dose-dependent manner. It was also observed that the more digested the samples were, the more inhibitory they were. Only a slight reduction in proliferation was seen after incubation of PBMCs with skim milk or bovine serum albumin. Further analysis revealed that the anti-proliferative effect of the whey protein samples was not caused by a toxic effect to

the cells. In addition, a flow cytometric apoptosis test indicated that the protein samples did not increase the number of apoptotic cells. The apoptosis test only shows an effect on the early event in the induction of apoptosis where phosphatidylserine is translocated to the outer part of the plasma membrane [229]. It was, however, not shown if the samples would have affected apoptosis in the cells at a later stage.

PBMCs are composed of many cell types such as T and B lymphocytes, monocytes and dendritic cells. In an attempt to identify which cell type was affected by the observed inhibitory effect, a population of T cells was generated (paper IV and V). When restimulating T cells with anti-CD3 coated beads in the presence of the whey protein samples the inhibitory effect on cell proliferation seemed enhanced. The most suppressive sample was the WPCG digested by both gastric and duodenal enzymes (paper IV and V). It was therefore suggested that the observed anti-proliferative effect on PBMCs was caused by one or several milk protein-derived components (proteins or peptides) through an interference with the generation of T cell activation signals (paper IV).

From the 109 peptides identified after simulated gastroduodenal digestion, 22 peptides originating form β-LG, β-CN and κ-CN glycomacropeptide were synthesised. The selected peptides cover large parts of each proteins primary sequence. In paper V hydrolysed caprine whey protein concentrate, fractions of this hydrolysate (> 8 kDa, 5-8 kDa and < 5 kDa, figure 13) and the single synthetic peptides identified in the fractions were screened for their effects on *in vitro* cell proliferation and cytokine production.

The synthetic peptides displayed variable effects on T-cell proliferation and none of the peptides that displayed suppressive effects were as inhibitory as the digested WPCG. This may indicate that we have either not tested the most suppressive componenent in the digested WPCG or that some of the peptides and proteins have synergistic effects.

The mitogen-induced proliferation assay is frequently used as a tool to rapidly screen for immunological effects [182, 188, 230]. Nevertheless, this test system does not discriminate between inhibition of early and later phases of the T-cell activation process. We therefore wanted to investigate how the cytokine secretion by T cells and dendritic cells were influenced by our samples.

Peripheral blood mononuclear cells are composed of various leukocytes such as lymphocytes, monocytes and dendritic cells. Screening for effects on these cell types individually would better elucidate the effects of the peptides.

The secretion of the following cytokines was studied: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, MCP-1 and IFN-γ. IL-2 is produced by T helper cells and is an important cytokine for growth and differentiation of T lymphocytes. MCP-1 is a strong chemokine recruiting both monocytes and T cells to the site of injury [231]. IL-8 is

considered a pro-inflammatory cytokine. It functions as a chemoattractant for neutrophils and activates these cells so that they degranulate and cause tissue damage [232]. Another pro-inflammatory cytokine is IL-1β which increases the production of endothelial adhesion molecules needed for leukocyte adhesion [232]. IL-10, an anti-inflammatory cytokine produced by Th2 cells suppresses the production of IL-1 and IL-8 [232]. IL-4, IL-5, IL-6 are Th2 cytokines and their secretion is thought to promote B cell proliferation and antibody secretion [67].

In general, the release of IL-1β and IL-12 by T cells was greatly increased by all the peptides, while the release of IL-4 and IL-5 was less influenced and sometimes even reduced. Secretion of IL-6, IL-8, IL-10, MCP-1 and IFN-γ was moderately stimulated by the synthetic peptides. Only the DC secretion of IL-6 and IL-8 was affected by the peptides in this study.

Most of the cytokines stimulated by the whey peptide samples are pro-inflammatory [232]. Nevertheless, a substantially increased secretion of IL-10 could indicate that the whey peptides might have a regulatory function. In addition, it has been shown that GMP could induce the expression of an IL-1 receptor antagonist (IL-1ra) [190] that could block the action of the pro-inflammatory IL-1 by binding of the IL-1ra to IL-1 receptors. GMP has by Requena *et al*. [192] also been shown to up-regulate the expression of the T regulatory cell marker forhead box P3 (Foxp3).

It was also observed that many of the samples tested increased the secretion of IL-2 and IFN-γ, important early signals in T-cell activation, but at the same time inhibited proliferation of activated T cells. If a downregulation in the IL-2 receptor, as seen for GMP [191] , occurs at the same time as IL-2 secretion is increased, T-cell proliferation could still be inhibited.

It is important to stress that only a small selection of the many cytokines that are produced by the immune cells have been included in the present study. The cytokine profiles of the tested peptides nevertheless seemed unique for each peptide and the many peptides present in the hydrolysates could display possible synergistic or antagonistic effects. The observed immunomodulatory effect could also possibly be receptor mediated either by binding to surface molecules on the cells or by binding to intracellular molecules. This, however, remains to be studied.

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This study has described individual differences in secretory volumes of the digestive juices, as well as variation in pH, enzyme activities and bile acids. It was stressed that human digestive juices would likely better mimic *in vivo* digestion due to their complex composition compared to the use of purified commercial enzymes. In addition, differences in gastric pH levels should be considered when performing such studies. The activities of pepsin and lipase decreased during storage and thus gastric and duodenal juice should be used within two and four months, respectively.

Digestion of whey proteins by human gastric and duodenal juices or commercial enzymes used at similar activities resulted in a more extensive hydrolysis by the latter.

Finally, it was described how different peptides identified within a hydrolysed whey protein concentrate could modulate immune responses by reducing the proliferation of T cells and by affecting the secretion of many cytokines by both T cells (adaptive immune system) and dendritic cells (innate immune system).

Future work should therefore:

- Take into consideration individual differences in volumes, gastric pH (especially adults and infants) and enzyme activities when performing *in vitro* digestion. Different amount of juices added to the substrate should be considered.

- *In vivo* studies should be performed to validate *in vitro* digestion models if possible.

- Digestion of pure proteins rather than a mixture could improve the detection of peptides.

- Experiments should be performed to investigate whether the observed antiproliferative effect on T cells was mediated by peptides binding to specific receptor on cell surfaces

- Synergistic effects of the synthetic peptides should also be studied.

6. APPENDICES

Appendix 1. Peptides originating from β-lactoglobulin produced after *in vitro* early digestion of WPCG with human gastroduodenal juices or commercial porcine digestive enzymes at (A) pH 2, (B) pH 4 and (C) pH 6 in the first step simulating gastric digestion. Red amino acids in the protein sequence indicate amino acids that are part of identified peptides.

(A)

Human gastric and duodenal juices pH 2					Porcine pepsin and pancreatic enzymes pH 2				
Position	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**	**Position**	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**
1 - 8	467,28	932,55	932,54	IIVTQTMK	1 - 8	467,26	932,50	932,54	IIVTQTMK
1 - 11	609,85	1217,69	1217,67	IIVTQTMKGLD	1 - 32	1146,57	3436,69	3436,83	IIVTQTMKGLDIQKVAGTWYSLAMAASDISLL
1 - 13	730,42	1458,83	1458,81	IIVTQTMKGLDIQ	9 - 19	594,28	1186,55	1186,63	GLDIQKVAGTW
1 - 14	794,45	1586,89	1586,91	IIVTQTMKGLDIQK	9 - 20	675,84	1349,66	1349,70	GLDIQKVAGTWY
1 - 20	755,73	2264,18	2264,22	IIVTQTMKGLDIQKVAGTWY	21 - 32	596,30	1190,59	1190,62	SLAMAASDISLL
9 - 20	675,85	1349,68	1349,70	GLDIQKVAGTWY	27 - 39	665,32	1328,62	1328,68	SDISLLDAQSAPL
21 - 32	596,33	1190,64	1190,62	SLAMAASDISLL	27 - 40	743,38	1484,74	1484,78	SDISLLDAQSAPLR
33 - 42	560,30	1118,58	1118,57	DAQSAPLRVY	27 - 41	792,87	1583,73	1583,85	SDISLLDAQSAPLRV
42 - 53	688,36	1374,71	1374,67	YVEELKPTPEGN	33 - 40	429,21	856,40	856,44	DAQSAPLR
42 - 57	922,48	1842,94	1842,96	YVEELKPTPEGNLEIL	41 - 56	915,45	1828,88	1828,95	VYVEELKPTPEGNLEI
43 - 55	727,85	1453,69	1453,73	VEELKPTPEGNLE	41 - 57	971,99	1941,96	1942,03	VYVEELKPTPEGNLEIL
43 - 57	840,97	1679,93	1679,90	VEELKPTPEGNLEIL	41 - 69	771,40	2311,18	2311,27	VYVEELKPTPEGNLEILLQK
43 - 58	897,48	1792,94	1792,98	VEELKPTPEGNLEILL	41 - 69	771,39	2311,13	2311,27	VYVEELKPTPEGNLEILLQK
43 - 59	961,56	1921,10	1921,04	VEELKPTPEGNLEILLQ	42 - 56	865,91	1729,81	1729,88	YVEELKPTPEGNLEI
83 - 95	734,92	1467,83	1467,87	KIDALNENKVLVL	42 - 57	922,46	1842,90	1842,96	YVEELKPTPEGNLEIL
149 - 159	609,32	1216,62	1216,61	LAFNPTQLEGQ	43 - 54	663,29	1324,56	1324,69	VEELKPTPEGNL
149 - 162	778,89	1555,77	1555,75	LAFNPTQLEGQCHV	43 - 55	727,83	1453,65	1453,73	VEELKPTPEGNLE
					43 - 56	784,36	1566,70	1566,81	VEELKPTPEGNLEI
					43 - 57	840,93	1679,85	1679,90	VEELKPTPEGNLEIL
					43 - 58	897,47	1792,92	1792,98	VEELKPTPEGNLEILL
					71 - 82	665,39	1328,76	1328,81	IIAEKTKIPAVF
					83 - 91	522,77	1043,52	1043,56	KIDALNENK
					83 - 91	522,77	1043,52	1043,56	KIDALNENK
					83 - 93	628,81	1255,60	1255,71	KIDALNENKVL
					83 - 99	654,99	1961,95	1962,03	KIDALNENKVLVLDTDY
					83 - 101	555,54	2218,11	2218,22	KIDALNENKVLVLDTDYKK
					84 - 91	458,72	915,43	915,47	IDALNENK
					125 - 134	565,76	1129,50	1129,55	TPEVDKEALE
					125 - 135	420,20	1257,59	1257,65	TPEVDKEALEK
					125 - 138	550,26	1647,77	1647,84	TPEVDKEALEKFDK
					125 - 139	573,94	1718,80	1718,87	TPEVDKEALEKFDKA
					142 - 148	419,23	836,44	836,47	ALPMHIR
					149 - 159	609,26	1216,51	1216,61	LAFNPTQLEGQ
					149 - 162	778,86	1555,70	1555,75	LAFNPTQLEGQCHV

Sequence coverage: 53 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEI
LLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSA
EPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

Sequence coverage: 78 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKW
ENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCL
VRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

(B)

Human gastric and duodenal juices pH 4					Porcine pepsin and pancreatic enzymes pH 4				
Position	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**	**Position**	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**
1 - 10	552,32	1102,62	1102,64	IIVTQTMKGL	1 - 13	730,36	1458,71	1458,81	IIVTQTMKGLDIQ
1 - 13	730,39	1458,77	1458,81	IIVTQTMKGLDIQ	1 - 14	529,96	1586,87	1586,91	IIVTQTMKGLDIQK
1 - 19	701,36	2101,07	2101,16	IIVTQTMKGLDIQKVAGTW	1 - 15	562,95	1685,84	1685,97	IIVTQTMKGLDIQKV
1 - 20	755,70	2264,07	2264,22	IIVTQTMKGLDIQKVAGTWY	1 - 19	701,36	2101,06	2101,16	IIVTQTMKGLDIQKVAGTW
6 - 18	681,32	1360,63	1360,74	TMKGLDIQKVAGT	1 - 20	755,71	2264,11	2264,22	IIVTQTMKGLDIQKVAGTWY
21 - 32	596,30	1190,59	1190,62	SLAMAASDISLL	1 - 29	1042,21	3123,60	3123,63	IIVTQTMKGLDIQKVAGTWYSLAMAASDI
33 - 42	560,28	1118,54	1118,57	DAQSAPLRVY	1 - 32	1146,60	3436,77	3436,83	IIVTQTMKGLDIQKVAGTWYSLAMAASDISLL
43 - 55	727,86	1453,70	1453,73	VEELKPTPEGNLE	9 - 19	594,29	1186,56	1186,63	GLDIQKVAGTW
43 - 57	840,90	1679,78	1679,90	VEELKPTPEGNLEIL	9 - 20	675,85	1349,68	1349,70	GLDIQKVAGTWY
43 - 58	897,42	1792,82	1792,98	VEELKPTPEGNLEILL	27 - 39	665,31	1328,61	1328,68	SDISLLDAQSAPL
43 - 59	641,32	1920,95	1921,04	VEELKPTPEGNLEILLQ	27 - 41	792,89	1583,76	1583,85	SDISLLDAQSAPLRV
83 - 93	628,82	1255,63	1255,71	KIDALNENKVL	33 - 40	429,22	856,42	856,44	DAQSAPLR
83 - 103	624,56	2494,23	2494,37	KIDALNENKVLVLDTDYKKYL	33 - 42	560,28	1118,55	1118,57	DAQSAPLRVY
135 - 145	421,24	1260,68	1260,73	KFDKALKALPM	33 - 44	674,35	1346,68	1346,68	DAQSAPLRVYVE
					33 - 57	927,81	2780,42	2780,46	DAQSAPLRVYVEELKPTPEGNLEIL
					42 - 57	922,48	1842,94	1842,96	YVEELKPTPEGNLEIL
					71 - 82	665,40	1328,79	1328,81	IIAEKTKIPAVF
					142 - 148	419,24	836,46	836,47	ALPMHIR

Sequence coverage: 56 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEI
LLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSA
EPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

Sequence coverage: 47 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKW
ENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCL
VRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

(C)

Human gastric and duodenal juices pH 6					Porcine pepsin and pancreatic enzymes pH 6				
Position	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**	**Position**	**Observed**	**Mr(expt)**	**Mr(calc)**	**Sequence**
1 - 18	639,33	1914,98	1915,08	IIVTQTMKGLDIQKVAGT	1 - 8	467,26	932,50	932,54	IIVTQTMK
1 - 20	1133,08	2264,14	2264,22	IIVTQTMKGLDIQKVAGTWY	9 - 19	594,31	1186,61	1186,63	GLDIQKVAGTW
1 - 26	937,13	2808,38	2808,49	IIVTQTMKGLDIQKVAGTWYSLAMAA	9 - 20	675,83	1349,65	1349,70	GLDIQKVAGTWY
21 - 32	596,30	1190,58	1190,62	SLAMAASDISLL	21 - 31	539,77	1077,52	1077,54	SLAMAASDISL
33 - 42	560,27	1118,53	1118,57	DAQSAPLRVY	21 - 32	596,31	1190,61	1190,62	SLAMAASDISLL
33 - 57	927,79	2780,35	2780,46	DAQSAPLRVYVEELKPTPEGNLEIL	27 - 41	792,91	1583,81	1583,85	SDISLLDAQSAPLRV
43 - 55	727,85	1453,68	1453,73	VEELKPTPEGNLE	83 - 93	628,84	1255,67	1255,71	KIDALNENKVL
43 - 56	784,37	1566,73	1566,81	VEELKPTPEGNLEI					
43 - 57	840,93	1679,84	1679,90	VEELKPTPEGNLEIL					
43 - 58	897,45	1792,88	1792,98	VEELKPTPEGNLEILL					
43 - 59	961,47	1920,93	1921,04	VEELKPTPEGNLEILLQ					
83 - 93	628,84	1255,67	1255,71	KIDALNENKVL					
94 - 103	629,31	1256,61	1256,67	VLDTDYKKYL					

Sequence coverage: 49 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEI
LLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSA
EPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

Sequence coverage: 32 %

IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKW
ENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCL
VRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV

Appendix 2. Total amount of peptides identified after *in vitro* digestion of caprine whey proteins with (A) gastric juice or (B) with gastric and duodenal juice.

(A)

Protein	Position	Observed	Mr(expt)	Mr(calc)	ppm	Sequence	> 8 kDa	< 8 kDa
β-LG	1-11	609,8	1217,6	1217,7	-87	IIVTQTMKGLD	x	x
	10-19	565,8	1129,6	1129,6	-56	LDIQKVAGTW	x	
	29-41	691,9	1381,7	1381,8	-40	ISLLDAQSAPLRV	x	x
	33-41	478,7	955,4	955,5	-80	DAQSAPLRV		x
	42-54	744,8	1487,6	1487,8	-73	YVEELKPTPEGNL		x
	42-55	809,4	1616,7	1616,8	-43	YVEELKPTPEGNLE		x
	42-57	615,3	1842,9	1843,0	-50	YVEELKPTPEGNLEIL	x	x
	43-57	840,9	1679,8	1679,9	-62	VEELKPTPEGNLEIL		x
	45-54	549,3	1096,5	1096,6	-53	ELKPTPEGNL		x
	83-95	734,9	1467,7	1467,9	-84	KIDALNENKVLVL		x
	87-95	521,3	1040,6	1040,6	-64	LNENKVLVL	x	
	142-149	475,7	949,5	949,6	-82	ALPMHIRL		x
κ-CN	106 - 113	450,2	898,4	898,5	-66	MAIPPKKD		x
	106 - 115	571,8	1141,5	1141,6	-64	MAIPPKKDQD		x
	106 - 124	1055,5	2108,9	2109,1	-100	MAIPPKKDQDKTEIPAINT	x	x
	106 - 126	1147,5	2293,1	2293,2	-77	MAIPPKKDQDKTEIPAINTIA		x
	106 - 139	905,2	3616,7	3616,8	-48	MAIPPKKDQDKTEVPAINTIASAEPTVHSTPTTE	x	
	106 - 139	908,7	3630,7	3630,8	-48	MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTE	x	
	106 - 139	909,2	3632,6	3632,8	-39	MAIPPKKDQDKTEDPAINTIASAEPTVHSTPTTE	x	
	106 - 139	912,7	3646,7	3646,8	-38	MAIPPKKDQDKTEEPAINTIASAEPTVHSTPTTE	x	
	107 - 115	506,2	1010,5	1010,5	-67	AIPPKKDQD		x
	109 - 132	646,0	2580,1	2580,3	-73	PPKKDQDKTEMPAINTIASAEPTV		x
	113 - 124	672,8	1343,6	1343,7	-45	DQDKTEIPAINT		x
	116 - 126	585,8	1169,6	1169,7	-85	KTEIPAINTIA	x	x
	124 - 139	821,4	1640,7	1640,8	-48	TIASAEPTVHSTPTTE		x
	125 - 139	770,8	1539,7	1539,7	-58	IASAEPTVHSTPTTE	x	x
	125 - 140	806,3	1610,6	1610,8	-94	IASAEPTVHSTPTTEA	x	
	126 - 139	714,3	1426,6	1426,7	-53	ASAEPTVHSTPTTE	x	
	127 - 138	614,3	1226,5	1226,6	-56	SAEPTVHSTPTT		x
	127 - 139	678,8	1355,5	1355,6	-78	SAEPTVHSTPTTE	x	x
	127 - 140	714,3	1426,5	1426,7	-91	SAEPTVHSTPTTEA		x
	128 - 139	635,3	1268,5	1268,6	-36	AEPTVHSTPTTE		x
	130 - 139	535,2	1068,4	1068,5	-58	PTVHSTPTTE	x	x
	130 - 140	570,7	1139,5	1139,5	-70	PTVHSTPTTEA	x	x
	140 - 163	1204,0	2405,9	2406,1	-87	AIVNTVDNPEASSESIASASETNT		x
	141 - 153	687,8	1373,6	1373,6	-37	IVNTVDNPEASSE		x
	141 - 154	731,3	1460,6	1460,7	-35	IVNTVDNPEASSES		x
	159 - 171	683,8	1365,6	1365,6	-35	SETNTAQVTSTEV	x	

(A) continued								
β-CN	1-6	402,2	802,3	802,4	-53	REQEEL		x
	1-8	508,7	1015,4	1015,5	-54	REQEELNV		x
	1-14	815,9	1629,7	1629,8	-47	REQEELNVVGETVE		x
	41-52	491,2	1470,6	1470,7	-57	TEDELQDKIHPF		x
	49-60	687,3	1372,6	1372,7	-54	IHPFAQAQSLVY	x	
	56-80	913,4	2737,2	2737,5	-97	QSLVYPFTGPIPNSLPQNILPLTQT		x
	58-75	992,0	1982,0	1982,1	-68	LVYPFTGPIPNSLPQNIL		x
	58-80	841,7	2522,2	2522,4	-82	LVYPFTGPIPNSLPQNILPLTQT	x	x
	59-71	701,3	1400,6	1400,7	-73	VYPFTGPIPNSLP		x
	59-75	935,4	1868,8	1869,0	-93	VYPFTGPIPNSLPQNIL	x	x
	59-80	1205,6	2409,1	2409,3	-69	VYPFTGPIPNSLPQNILPLTQT	x	x
	61-75	804,4	1606,8	1606,9	-72	PFTGPIPNSLPQNIL	x	x
	61-80	716,7	2147,0	2147,2	-69	PFTGPIPNSLPQNILPLTQT	x	x
	76-97	796,4	2386,2	2386,3	-45	PLTQTPVVVPPFLQPEIMGVPK	x	
	81-89	498,3	994,5	994,6	-74	PVVVPPFLQ		x
	81-92	667,9	1333,7	1333,8	-54	PVVVPPFLQPEI	x	x
	81-93	733,4	1464,7	1464,8	-56	PVVVPPFLQPEIM	x	x
	81-94	761,9	1521,7	1521,8	-66	PVVVPPFLQPEIMG		x
	81-96	859,9	1717,8	1717,9	-67	PVVVPPFLQPEIMGVP		x
	81-97	924,0	1845,9	1846,0	-79	PVVVPPFLQPEIMGVPK	x	x
	81-99	692,0	2073,1	2073,2	-52	PVVVPPFLQPEIMGVPKVK	x	x
	81-100	735,0	2202,1	2202,2	-77	PVVVPPFLQPEIMGVPKVKE	x	x
	81-105	920,5	2758,4	2758,6	-42	PVVVPPFLQPEIMGVPKVKETMVPK	x	
	81-106	724,9	2895,4	2895,6	-73	PVVVPPFLQPEIMGVPKVKETMVPKH		x
	94-105	438,2	1311,7	1311,8	-61	GVPKVKETMVPK		x
	96-107	474,6	1420,7	1420,8	-62	PKVKETMVPKHK		x
	98-113	482,2	1924,9	1925,0	-79	VKETMVPKHKEMPFPK		x
	127-141	572,3	1713,9	1714,0	-63	LTDVEKLHLPLPLVQ	x	x
	128-141	801,4	1600,8	1600,9	-61	TDVEKLHLPLPLVQ	x	x
	128-142	563,6	1687,8	1688,0	-79	TDVEKLHLPLPLVQS		x
	129-141	500,9	1499,8	1499,9	-71	DVEKLHLPLPLVQ		x
	130-141	462,6	1384,7	1384,8	-68	VEKLHLPLPLVQ	x	
	142-156	868,3	1734,7	1734,8	-87	SWMHQPPQPLSPTVM		x
	142-163	835,4	2503,0	2503,2	-80	SWMHQPPQPLSPTVMFPPQSVL	x	x
	164-181	500,8	1999,1	1999,2	-69	SLSQPKVLPVPQKVVPQR		x
	164-187	885,8	2654,4	2654,5	-46	SLSQPKVLPVPQKVVPQRDMPIQA	x	
	164-188	701,4	2801,4	2801,6	-69	SLSQPKVLPVPQKVVPQRDMPIQAF		x
	170-187	672,3	2014,0	2014,1	-95	VLPVPQKVVPQRDMPIQA		x
	191-205	834,9	1667,8	1667,9	-69	YQEPVLGPVRGPFPI	x	x
	191-206	891,4	1780,8	1781,0	-84	YQEPVLGPVRGPFPIL	x	x
	191-207	940,9	1879,9	1880,1	-100	YQEPVLGPVRGPFPILV		x
	194-205	624,8	1247,6	1247,7	-92	PVLGPVRGPFPI		x
	194-206	681,4	1360,7	1360,8	-89	PVLGPVRGPFPIL		x
	198-206	498,3	994,5	994,6	-99	PVRGPFPIL		x

(A) continued								
BSA	1 - 17	666,6	1996,8	1996,9	-57	DTHKSEIAHRFNDLGEE		x
	1 - 21	815,3	2442,9	2443,1	-83	DTHKSEIAHRFNDLGEENFQG		x
	15 - 24	553,3	1104,5	1104,6	-37	GEENFQGLVL		x
	71 - 83	717,8	1433,6	1433,7	-91	GDELCKVATLRET		x
	228 - 237	552,8	1103,6	1103,6	-40	TDVTKIVTDL		x
	491 - 505	849,3	1696,6	1696,8	-88	TLDETYVPKPFDGES		x
	493 - 503	634,3	1266,5	1266,6	-61	DETYVPKPFDG		x
	493 - 505	742,3	1482,5	1482,7	-93	DETYVPKPFDGES		x
	493 - 507	866,3	1730,7	1730,8	-68	DETYVPKPFDGESFT		x
(A) continued								
LF	98 - 111	812,3	1622,6	1622,8	-78	AEIYGTEKSPQTHY	x	x
	143 - 154	684,8	1367,7	1367,8	-64	GWNIPVGILRPF		x
	159 - 171	687,8	1373,6	1373,7	-53	ESAEPLQGAVARF		x
	160 - 171	623,3	1244,5	1244,7	-96	SAEPLQGAVARF	x	x
	228 - 237	590,3	1178,6	1178,6	-55	VKETTVFENL		x
	235 - 246	493,2	1476,6	1476,7	-48	ENLPEKADRDQY	x	
	277 - 285	509,2	1016,5	1016,5	-50	RSVDGKENL	x	
	290 - 307	552,3	2205,1	2205,2	-83	LRKAQEKFGKNKSQRFQL		x
	327 - 336	536,3	1070,6	1070,6	-52	VRIPSKVDSA		x
	698 - 708	617,3	1232,6	1232,7	-79	PLLEACAFLTR		x

(B)

Protein	Position	Observed	Mr(expt)	Mr(calc)	ppm	Sequence	> 8 kDa	5-8 kDa	< 5 kDa
β-LG	1-8	467,3	932,5	932,5	-47	IIVTQTMK	x		
	1-13	730,4	1458,8	1458,8	-46	IIVTQTMKGLDIQ	x		
	1-14	529,9	1586,8	1586,9	-86	IIVTQTMKGLDIQK	x		
	1-20	755,7	2264,1	2264,2	-50	IIVTQTMKGLDIQKVAGTWY	x		
	1-28	1004,5	3010,4	3010,6	-43	IIVTQTMKGLDIQKVAGTWYSLAMAASD	x		
	1-29	1042,2	3123,5	3123,6	-43	IIVTQTMKGLDIQKVAGTWYSLAMAASDI	x		
	1-32	1146,6	3436,7	3436,8	-47	IIVTQTMKGLDIQKVAGTWYSLAMAASDISLL	x		
	9-18	501,2	1000,5	1000,6	-80	GLDIQKVAGT			x
	9-20	675,8	1349,6	1349,7	-56	GLDIQKVAGTWY	x		
	9-23	811,4	1620,8	1620,9	-41	GLDIQKVAGTWYSLA	x		
	9-25	912,4	1822,9	1822,9	-39	GLDIQKVAGTWYSLAMA	x		
	9-26	948,0	1893,9	1894,0	-37	GLDIQKVAGTWYSLAMAA	x		
	21-32	596,3	1190,6	1190,6	-41	SLAMAASDISLL	x		
	33-39	429,2	856,4	856,4	-53	DAQSAPLR	x		
	33-41	478,7	955,4	955,5	-76	DAQSAPLRV			x
	33-42	560,3	1118,5	1118,6	-44	DAQSAPLRVY	x		
	33-57	927,8	2780,3	2780,5	-46	DAQSAPLRVYVEELKPTPEGNLEIL	x		
	36-59	903,5	2707,4	2707,5	-44	SAPLRVYVEELKPTPEGNLEILLQ	x		
	40-59	780,7	2339,2	2339,3	-43	RVYVEELKPTPEGNLEILLQ	x		
	41-56	915,4	1828,8	1828,9	-64	VYVEELKPTPEGNLEI			x
	41-57	972,0	1941,9	1942,0	-49	VYVEELKPTPEGNLEIL			x
	41-59	1092,6	2183,1	2183,2	-42	VYVEELKPTPEGNLEILLQ	x		
	42-54	744,8	1487,6	1487,8	-77	YVEELKPTPEGNL		x	
	42-56	865,9	1729,8	1729,9	-52	YVEELKPTPEGNLEI		x	x
	43-55	727,8	1453,6	1453,7	-88	VEELKPTPEGNLE			x
	43-56	784,4	1566,7	1566,8	-71	VEELKPTPEGNLEI		x	x
	43-57	840,9	1679,8	1679,9	-44	VEELKPTPEGNLEIL	x	x	x
	43-59	961,5	1921,0	1921,0	-41	VEELKPTPEGNLEILLQ	x		
	71-82	443,9	1328,7	1328,8	-57	IIAEKTKIPAVF	x		x
	83-91	522,8	1043,5	1043,6	-58	KIDALNENK			x
	83-93	628,8	1255,6	1255,7	-55	KIDALNENKVL		x	
	88-98	630,3	1258,6	1258,6	-50	NENKVLVLDTD			x
	92-100	533,3	1064,5	1064,6	-50	VLVLDTDYK			x
	123-137	592,6	1774,8	1774,9	-83	VRTPEVDKEALEKFD		x	
	125-134	565,7	1129,5	1129,6	-85	TPEVDKEALE		x	
	125-135	420,2	1257,5	1257,6	-92	TPEVDKEALEK		x	
	125-136	469,2	1404,6	1404,7	-70	TPEVDKEALEKF		x	
	125-137	507,5	1519,6	1519,7	-85	TPEVDKEALEKFD		x	x
	125-138	550,2	1647,7	1647,8	-86	TPEVDKEALEKFDK	x	x	x
	125-147	656,6	2622,3	2622,4	-54	TPEVDKEALEKFDKALKALPMHI	x		
	131-138	490,2	978,5	978,5	-52	EALEKFDK	x		
	139-147	497,3	992,5	992,6	-80	ALKALPMHI	x		
	149-159	609,3	1216,5	1216,6	-53	LAFNPTQLEGQ		x	x

(B) continued								
κ-CN	106 - 124	704,0	2109,0	2109,1	-60	MAIPPKKDQDKTEIPAINT		x
	106 - 125	556,5	2222,1	2222,2	-56	MAIPPKKDQDKTEIPAINTI		x
	106 - 126	765,4	2293,1	2293,2	-58	MAIPPKKDQDKTEIPAINTIA		x
	109 - 121	484,9	1451,7	1451,8	-59	PPKKDQDKTEVPA	x	x
	109 - 122	532,6	1594,8	1594,8	-42	PPKKDQDKTEEPAI		x
	109 - 125	482,2	1924,9	1925,0	-44	PPKKDQDKTEMPAINTI		x
	109 - 126	660,3	1978,0	1978,1	-58	PPKKDQDKTEIPAINTIA		x
	110 - 122	489,6	1465,7	1465,8	-58	PKKDQDKTEPPAI	x	
	113 - 121	508,7	1015,4	1015,5	-73	DQDKTEIPA	x	x
	113 - 123	622,3	1242,5	1242,6	-70	DQDKTEIPAIN	x	
	113 - 124	672,8	1343,6	1343,7	-60	DQDKTEIPAINT	x	x
	113 - 125	729,3	1456,7	1456,7	-61	DQDKTEIPAINTI	x	
	113 - 126	764,8	1527,6	1527,8	-97	DQDKTEIPAINTIA	x	
	122 - 133	626,8	1251,6	1251,6	-68	INTIASAEPTVH		x
	124 - 133	513,2	1024,5	1024,5	-67	TIASAEPTVH		x
	125 - 139	770,8	1539,6	1539,7	-95	IASAEPTVHSTPTTE	x	
	125 -140	806,3	1610,7	1610,8	-70	IASAEPTVHSTPTTEA	x	
	126 - 133	406,2	810,3	810,4	-57	ASAEPTVH	x	x
	126 - 139	714,3	1426,6	1426,7	-55	ASAEPTVHSTPTTE	x	
	127 - 140	714,3	1426,6	1426,7	-65	SAEPTVHSTPTTEA	x	
	130 - 139	535,2	1068,4	1068,5	-57	PTVHSTPTTE	x	
	141 - 153	687,8	1373,6	1373,6	-52	IVNTVDNPEASSE	x	
	143 - 156	717,3	1432,5	1432,6	-57	NTVDNPEASSESIA	x	
(B) continued								
β-CN	1-9	558,3	1114,5	1114,6	-62	REQEELNVV		x
	41-51	662,8	1323,5	1323,6	-76	TEDELQDKIHP		x
	41-52	736,3	1470,6	1470,7	-63	TEDELQDKIHPF		x
	57-68	652,8	1303,6	1303,7	-44	SLVYPFTGPIPN		x
	57-76	1084,0	2166,0	2166,2	-63	SLVYPFTGPIPNSLPQNILP		
	57-77	1140,6	2279,1	2279,3	-73	SLVYPFTGPIPNSLPQNILPL	x	x
	59-72	765,4	1528,7	1528,8	-48	VYPFTGPIPNSLPQ		x
	59-77	1040,5	2079,0	2079,1	-70	VYPFTGPIPNSLPQNILPL	x	
	61-72	634,3	1266,6	1266,7	-67	PFTGPIPNSLPQ		x
	61-77	909,5	1816,9	1817,0	-41	PFTGPIPNSLPQNILPL	x	x
	63-77	787,4	1572,8	1572,9	-70	TGPIPNSLPQNILPL		x
	69-92	880,8	2639,3	2639,5	-66	SLPQNILPLTQTPVVVPPFLQPEI	x	
	73-92	1108,1	2214,1	2214,3	-64	NILPLTQTPVVVPPFLQPEI	x	
	78-91	776,4	1550,8	1550,8	-40	TQTPVVVPPFLQPE	x	x
	78-92	832,9	1663,8	1663,9	-49	TQTPVVVPPFLQPEI	x	x
	78-93	898,4	1794,9	1795,0	-51	TQTPVVVPPFLQPEIM	x	x
	81-91	611,3	1220,6	1220,7	-75	PVVVPPFLQPE	x	x
	81-92	667,9	1333,7	1333,8	-41	PVVVPPFLQPEI	x	x
	81-93	733,4	1464,7	1464,8	-39	PVVVPPFLQPEIM	x	x
	99-105	416,7	831,4	831,5	-52	KETMVPK		x
	106-119	582,6	1744,8	1744,9	-55	HKEMPFPKYPVEPF		x
	144-151	474,2	946,4	946,5	-71	MHQPPQPL		x
	144-154	616,8	1231,5	1231,6	-69	MHQPPQPLSPT		x
	170-180	602,3	1202,7	1202,7	-69	VLPVPQKVVPQ		x
	191-205	834,9	1667,8	1667,9	-64	YQEPVLGPVRGPFPI		x

(B) continued								
α-LA	12 - 27	909,9	1817,9	1818,0	-47	LKDLKDYGGVSLPEWV		x
	14 - 25	646,8	1291,5	1291,6	-80	DLKDYGGVSLPE	x	
	14 - 26	739,8	1477,7	1477,7	-38	DLKDYGGVSLPEW	x	
	14 - 27	789,4	1576,7	1576,8	-34	DLKDYGGVSLPEWV	x	
	16 - 27	675,3	1348,6	1348,7	-42	KDYGGVSLPEWV	x	
	17 - 27	611,3	1220,5	1220,6	-42	DYGGVSLPEWV	x	
	40 - 51	655,8	1309,5	1309,6	-55	AIVQNNDSTEYG	x	
BSA	10 - 18	547,2	1092,4	1092,5	-53	RFNDLGEEN	x	x
	10 - 19	620,8	1239,5	1239,6	-42	RFNDLGEENF		x
	10 - 21	713,3	1424,5	1424,6	-81	RFNDLGEENFQG	x	x
	11 - 21	635,2	1268,4	1268,5	-68	FNDLGEENFQG	x	
	493 - 501	548,2	1094,5	1094,5	-54	DETYVPKPF	x	x
LF	145 - 154	563,3	1124,6	1124,7	-89	NIPVGILRPF		x
	160 - 171	623,3	1244,6	1244,7	-59	SAEPLQGAVARF	x	
	235 - 245	438,8	1313,5	1313,6	-89	ENLPEKADRDQ	x	
	235 - 247	536,2	1605,6	1605,7	-75	ENLPEKADRDQYE	x	
	238 - 245	479,7	957,4	957,5	-87	PEKADRDQ	x	
	316 - 325	539,8	1077,5	1077,6	-42	DLLFKDSALG		x

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8. ENCLOSED PAPERS I-V

Paper I

Food Dig.
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Human Gastrointestinal Juices Intended for Use in In Vitro Digestion Models

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Abstract The aim of this study was to characterise the individual human gastric and duodenal juices to be used in in vitro model digestion and to examine the storage stability of the enzymes. Gastroduodenal juices were aspirated, and individual variations in enzymatic activities as well as total volumes, pH, bile acids, protein and bilirubin concentrations were recorded. Individual pepsin activity in the gastric juice varied by a factor of 10, while individual total proteolytic activity in the duodenal juice varied by a factor of 5. The duodenal amylase activity varied from 0 to 52.6 U/ml, and the bile acid concentration varied from 0.9 to 4.5 mM. Pooled gastric and duodenal juices from 18 volunteers were characterised according to pepsin activity (26.7 U/ml), total proteolytic activity (14.8 U/ml), lipase activity (951.0 U/ml), amylase activity (26.8 U/ml) and bile acids (4.5 mM). Stability of the main enzymes in two frozen batches of either gastric or duodenal juice was studied for 6 months. Pepsin activity decreased rapidly and adjusting the pH of gastric juice to 4 did not protect the pepsin from degradation. Lipase activity remained stable for 4 months, however decreased rapidly thereafter even after the addition of protease inhibitors. Glycerol only marginally stabilised the survival of the enzymatic activities. These results of compositional variations in the individual gastrointestinal juices and the effect of storage conditions on enzyme activities are useful for the design of in vitro models enabling human digestive juices to simulate physiological digestion.

Keywords Gastric juice · Duodenal juice · Enzymes · Individual variation · Storage stability

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Introduction

Several approaches to mimic in vivo digestion have been suggested over the years. Some of the purposes have been to investigate whether food components could produce bioactive components [1, 2], to study the survival of drugs through the gastrointestinal (GI) tract [3, 4] or to predict the digestibility of food proteins including allergens [5–9].

A recent review on in vitro human digestion models by Hur et al. [10] demonstrates the vast variability of digestive fluids used and emphasises the importance of using physiologically relevant levels of enzymes and other minor components when designing these fluids. Most previously reported in vitro digestion models have been performed with commercial digestive enzymes of porcine or bovine origin [2, 11–14]. To increase lipolysis, bile salts of various concentrations have been added [10], and also proteolysis has been shown to increase upon the addition of bile salts [15]. It was recently demonstrated that whey proteins digested by human gastroduodenal

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juices produced different peptides compared to digestion with porcine digestive enzymes [16]. Human digestive juices contain enzymes of various isoforms that may differ from purified animal enzymes with regard to both specificity and activity [17–19]. Enzyme activities have been estimated by different methods in different studies; thus, the outcomes will vary and will rely on the assay conditions such as the pH, temperature, substrate, incubation time and the detection method used [10]. Consequently, a direct comparison between studies is challenging. Human digestive juices contain enzymes and cofactors in a physiological combination and may therefore represent an advantage over artificial solutions composed of purified commercial enzymes. However, all physiological mechanisms operative in vivo are not transplantable to any in vitro setting.

In order to reduce the inter-individual variations and to be able to perform several comparable in vitro gastrointestinal digestion assays, batches of pooled gastric and intestinal juices are required. The specific activities of the enzymes in such pooled samples must be analysed, and their stability during storage should be monitored so that the juices are used while the enzymes are still active. Loss of pepsin activity in frozen gastric juice has been reported [20]. However, de Gara et al. [20] showed that the activity could be preserved by the addition of glycerol or by increasing the pH outside of the optimal pH range for pepsin. The amylase activity in duodenal juice stored at −20 °C remained largely stable in a study by Muller and Ghale [21] whereas lipase and trypsin activities in hormonally stimulated juice decreased to approximately 50% of the initial activities within 3 weeks. Furthermore, it has been suggested that adding protease inhibitors would result in a protective effect on the lipase activity as chymotrypsin has been shown to digest the lipase [22]. Based on this knowledge, we designed the present study with the following aims:

1. To establish a practical and convenient method for the collection of human intestinal juices from the upper digestive tract from a number of volunteers
2. To describe the individual variations in enzymatic activities as well as total volumes, pH, bile acids, total protein and bilirubin concentrations, of gastric and duodenal juices
3. To study the stability of enzyme activities in gastric and duodenal juices using different storage temperatures (−20 °C, −80 °C or liquid nitrogen), pH adjustment or the addition of glycerol or protease inhibitors
4. To characterise the pooled samples of gastric and duodenal juices enabling several in vitro digestion experiments to be carried out under similar conditions

Methods

Collection of Physiological Gastric and Duodenal Juices from the Upper Digestive Tract

Human gastric (HGJ) and duodenal (HDJ) juices were collected according to Holm et al. [23] from healthy volunteers without previous history of health impairments. Aspirations of in total 20 fasting volunteers (7 men and 13 women) aged between 20 to 42 years (average 25±5 years) were performed at Moss Hospital. The volunteers were allowed to drink water ad libitum prior to the placement of the tube. The aspiration was approved by the Norwegian Ethics Committee, and all volunteers had given a written consent to participate in the study. GI juices were collected by placing a flexible three-lumen silicone tube (Fig. 1a) developed by Maxter Catheters (Marseilles, France) especially for this type of study through the nose or mouth of the volunteer. Correct placement of the tube was monitored by radiology (Fig. 1b). Continuous instillation, 100 ml/h of an isotonic stimulatory solution (17.5 g/l sucrose, 450 mg/l NaCl, 800 mg/l L-phenylalanine and 575 mg/l L-valine in H_2O) [24] and a continuous aspiration of gastric and duodenal fluids were performed simultaneously. The stimulation solution was instilled close to the papilla of Vater, while the duodenal juice was aspirated some 10 cm distally. Juices from the stomach were aspirated from the canalis ventriculi. The GI juices were collected in 50 ml tubes on ice, inspected and pH was measured periodically to avoid samples of mixed duodenal and gastric juices. Digestive juice collected from the stomach was discarded if the juice was yellow in colour and had a pH >4. These samples were found to have low pepsin activity and high total proteolytic activity. Similarly, digestive juice of clear colour and pH ≤4 collected from the duodenum was also discarded. These samples had low total proteolytic activity and high pepsin activity as a result of gastric juice leakage to the duodenum (results not shown). The experiment lasted approximately 2 h per individual. The samples were centrifuged (4,500×*g* for 10 min) to remove mucous, and cell debris before aliquots were frozen at −20 °C and then stored at −20 °C or −80 °C.

Outline of the Study

The study was designed in three parts

Part I: Recording of individual variations in enzymatic activities, bile acids, total protein and bilirubin concentrations as well as pH and total volumes of gastric and duodenal juices. Due to technical disorganisation of all the individual aspirates, some enzyme activities and component analysis are missing in the data set.

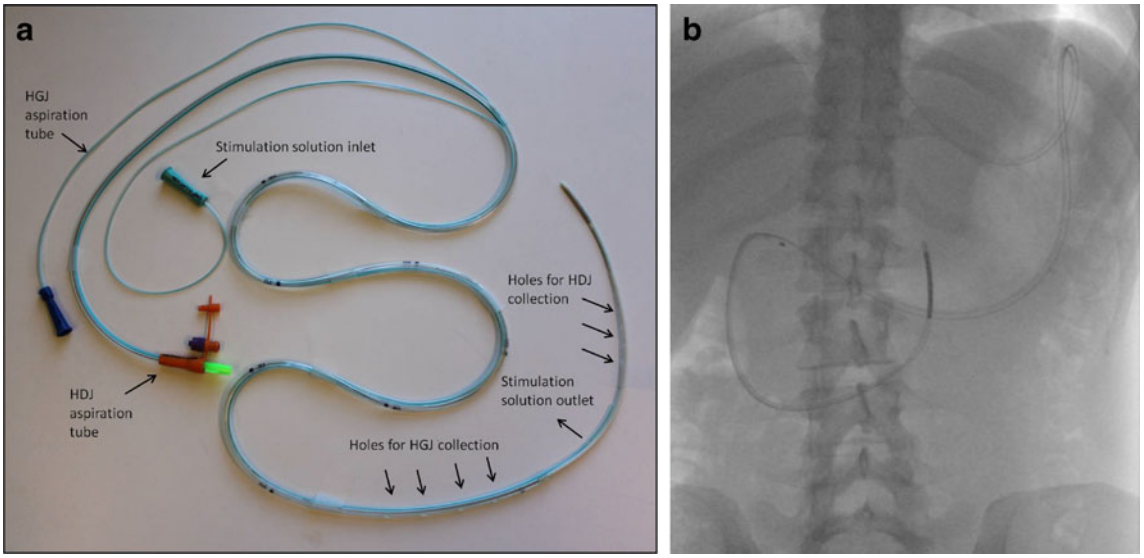

Fig. 1 **a** Detailed description of the three-lumen tube used for the aspiration of gastric and duodenal juices. *HGJ* gastric juice, *HDJ* duodenal juice (photo: Ellen K. Ulleberg). **b** Correct placement of the three-lumen aspiration tube in the stomach and duodenum of a volunteer as monitored by radiology (photo: Espen B. Herud)

Part II: Recording of storage stability. In order to examine the effect of different storage conditions on the stability of proteolytic, lipase and amylase activities, we pooled samples of gastric and duodenal juices, respectively, from two individuals (25 years old, one female and one male). The pooled gastric juice was divided into three subsamples: one control sample (no additions), one sample with glycerol added to a final concentration of 13% and one sample adjusted to pH 4 using a 0.2-M citrate buffer. The pooled duodenal juice was divided into seven subsamples: one control sample (no additions), one sample stored in liquid nitrogen (N_2, no additions), one sample with glycerol added to a final concentration of 13% and four samples with protease inhibitors added: (a) 29 mg/ml Pefabloc (Pentapharm Ltd, Basel, Switzerland), (b) 4 mg/ml Camostat mesilate (1879 China Langchem Inc., China), (c) 4 mg/ml Bowman Birk inhibitor (BBI; T9777, Sigma-Aldrich, St. Louis, MO, USA) or (d) a mixture of 4.5 mg/ml BBI and 34 mg/ml Pefabloc. All juices were aliquoted and stored at −80 °C unless otherwise is stated. The enzymatic activities were analysed on the day of collection and every month for 6 months.

Part III: Recording of changes in enzyme activities in pooled samples of HGJ and HDJ during long-term storage. For practical reasons, collection of a large number of samples may require a long period of time. Consequently, samples will have to be stored/frozen for different times before being pooled. To study this situation, samples of gastric and duodenal juices from 18 individuals (6 males and 12 females, average age 24±5 years) were pooled. The individual juices were collected and stored at −80 °C from 7 to 15 months prior to pooling them. The pooled juices were aliquoted and stored at −20 °C or at −80 °C. Registration of pH, volume and enzymatic activities as well as total bile acid, protein and bilirubin concentrations were performed on the day of making the pooled samples. Storage stability of the enzymatic activities in the frozen juices was then observed over a 12-month period after pooling the samples.

Proteolytic Activities of Gastric and Duodenal Enzymes

HGJ was analysed for pepsin activity at pH 3.0 with haemoglobin (H2625, Sigma, St. Louis, MO, USA) as substrate according to Sanchez-Chiang et al. [25]. Pepsin activity was assayed within 2 months after aspiration. HDJ was analysed for total proteolytic activity at pH 8.0 with casein (Hammarstein casein, Merck Co., Darmstadt, Germany) as substrate, as described by Krogdahl and Holm [26] and Kirschenbaum [27]. In brief, triplicates of human gastric or duodenal juices in three concentrations were incubated with substrate for 10 min at 37 °C, and the reactions were stopped by the addition of trichloroacetic acid. After an overnight sedimentation at 4 °C, the samples were centrifuged for 10 min at 3,000×*g*. One unit of enzyme activity was defined as the amount (in millilitres) of gastric or duodenal juice giving a difference in absorbance of 1.0 at 280 nm in 10 min at 37 °C.

Lipase Activity

Pancreatic lipase activity in the HDJ was measured in triplicates using the LIP, lipase colorimetric assay for serum analysis in automated systems, Roche Diagnostics GmbH (Mannheim, Germany). This assay is performed at pH 8 and uses the non-diglyceride chromogenic lipase substrate 1,2-*O*-dilauryl-rac-glycero-3-glutaric acid-(6-methylresorufin) ester. In brief, the samples were mixed with colipase in *N*,*N*-bis(2-hydroxyethyl)-glycine buffer, in a 96-well plate on ice and incubated at 37 °C for 5 min. After adding the substrate, the colour reaction was measured photometrically every 10 s for 5 min at 595 nm. The lipase cleaves the substrate to produce 1,2-*O*-dilauryl-rac-glycerol and the unstable glutaric acid-(6-methylresorufin) ester which then decomposes spontaneously producing the chromophore methylresorufin. The HDJ samples were diluted in 0.9% NaCl (1:10–1:100) depending on the activity of the juices. One unit of lipase activity was defined as the amount (in millilitres) of HDJ giving a difference in velocity (ΔOD, rate of methylresorufin production) of 1.0 at 595 nm/min at 37 °C.

Amylase Activity

HDJ was analysed for pancreatic amylase activity at Oestfold Hospital Trust using the ADVIA 1650 chemistry systems (Siemens Medical Solutions, Erlangen, Germany). The amylase in the sample splits the ethylidene-*p*-nitrophenyl-maltoheptaoside substrate, and together with added glucosidase, this leads to the release of free *p*-nitrophenol which is measured due to its absorbance at 410/694 nm. Two dilutions (1:5 and 1:10 in 0.9% NaCl) of the HDJ were made, and the average of the two were used to calculate the amylase activity. One unit of amylase is defined as that amount of HDJ, which catalyses the production of 1 μmol of *p*-nitrophenol per minute under the conditions of the method.

Bile Acids

Bile acid concentration was analysed in duplicates after dilution with distilled water (1:50) at the Central Laboratory of the Norwegian School of Veterinary Science (Oslo, Norway) using Advia® 1650 (Bayer Healthcare, Tarrytown, NY, USA), an automated analysis system for clinical chemistry. Principles of analyses were based on enzymatic amplification determining total 3α-hydroxy bile acids using a kit (Bio-stat Diagnostic systems, Stockport, UK). In the presence of Thio-NAD, the enzyme 3α-hydroxysteroid dehydrogenase converts bile acids to 3-keto steroids and Thio-NADH. The rate of formation of Thio-NADH was measured spectrophotometrically at 410 nm. The bile acid concentration was determined using a curve generated from a standardised taurocholic acid solution.

Total Protein Concentration

The total protein concentrations in the gastric and duodenal juices were measured in triplicates using the Qubit™ Fluorometer (Invitrogen) with the Quant-IT™ protein assay kit (Invitrogen) according to the manufacturer's instructions.

Bilirubin Concentration

Bilirubin concentration in the duodenal juices was analysed at Oestfold Hospital Trust using the ADVIA 1650 chemistry systems (Siemens Medical Solutions, Erlangen, Germany). Vanadate is used to oxidize bilirubin and thereby creating biliverdin. The optical density of the yellow colour of bilirubin (451/545 nm) is thereby decreased proportionally to the total bilirubin concentration in the duodenal juices. Two dilutions (1:5 and 1:10 in 0.9% NaCl) of the HDJ were made, and the average of the two were used to calculate the concentration of bilirubin.

Statistical Analysis

Normality of the individual data (volumes, total protein, enzyme activities, bile acids and bilirubin) was tested using the Shapiro–Wilk and the Kolmogorov–Smirnov tests using the SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Total volumes and pH of the gastric and duodenal juices collected from 18 volunteers over a 2-h period are presented in Table 1. The pH of the gastric juice varied from 1 to 4. The total volume of the individual gastric juices varied with a minimum of 2 ml and a maximum output of 355 ml, with an average of 87±103 ml. The total volume of duodenal juice for each individual ranged from 10 to 312 ml with an average of 158±91 ml and a pH between 5 and 9. A normal distribution of the data for HGJ volumes was only obtained after logarithmic transformation of the numbers, whereas the original data for HDJ volumes were normally distributed (data not shown). It was observed that for ten of the volunteers, substantial volumes of gastric juice (20 to 123 ml) seemed to leak and mix with the duodenal juice. Three of the volunteers also experienced reflux of duodenal juice (10 to 30 ml) to the gastric compartment (Table 1). These samples were discarded from further analysis.

Table 1 Total volumes and periodically measured pH during the collection of the gastric and duodenal juices from 18 individuals

Volunteer			Gastric juice			Duodenal juice		
No	Age	Gender	pH	Volume (ml)	–[a]	pH	Volume (ml)	–[b]
1	24	F	nd	65		nd	161	
2	21	F	4	13		7	27	
3	22	F	2	17	30	6–7	100	
4	42	F	1	355	50	5–9	205	
5	20	M	1–2	222	115	7–8	110	
6	20	F	2	55	37	6–8	155	
7	24	F	3–4	110		6–7	30	14
8	21	F	4	25		6	10	10
9	20	M	2	15	35	5–7	218	
10	30	F	1–4	198		6–7	30	
11	34	M	3–4	13		5–7	152	
12	21	F	1	28	123	7–8	197	
13	26	M	3–4	37		5–7	168	30
14	24	F	4	10	99	5–7	285	
15	22	M	4	31		5–7	253	
16	24	F	2–3	121	20	6–7	244	
17	24	F	1	2	73	6–7	312	
18	23	M	1	248	73	6–8	185	
Average	25			87			158	
SD	6			103			91	

F female, *nd* not determined, *M* male, *SD* standard deviation

[a]Additional volume aspirated from the duodenal tube with pH ≤4

[b]Additional volume aspirated from the gastric tube with pH >4

The individual differences in enzymatic activities and the concentrations of bile acids, total protein and bilirubin are described in Table 2. Data for the enzymatic activities, bile acids and total protein showed normal distribution, whereas bilirubin was only normally distributed if the very high concentration found for volunteer 17 was removed (data not shown). The pepsin activities of the gastric juices ranged from 7 to 70 U/ml, with an average activity of 37±21 U/ml. A high volume of HGJ did not result in a decreased pepsin activity or vice versa (r=−0.55). The total proteolytic activity of the individual duodenal juice did not vary to the same extent ranging from approximately 5 to 25 U/ml, with an average of 16±6 U/ml. The correlation between volume and total proteolytic activity was r=0.14. The duodenal amylase activity ranged from 0 to 50 U/ml with an average of 27±15 U/ml. A high variation in total bile acid and bilirubin concentrations was also observed with average values of 2.7 (±1.3) mM and 59 (±59) μM, respectively. Total protein concentrations in gastric and duodenal juice were 1.1 (±0.3) and 1.4 (±0.7) mg/ml, respectively. Correlations between gastric and duodenal output volumes and pepsin versus total proteolytic activity outputs were r=−0.10 and r=0.20, respectively.

Gastric and duodenal juices from two volunteers were pooled on the day of aspiration, and the stability of the enzymatic activities was monitored for 6 months. The stability of the pepsin activity in the gastric juice and the amylase, total proteolytic and lipase activities in the duodenal juice is illustrated in Fig. 2. Two months after aspiration, approximately 80% of the pepsin activity remained. Then, after 3 months, the activity seemed to stabilise at approximately 60% of the initial pepsin activity. The addition of glycerol or a pH adjustment of the gastric juice did not protect the pepsin from degradation (data not shown). The amylase activity in the pooled duodenal juice did not decrease during storage, and a decay of only approximately 10% total proteolytic activity was observed. No decrease in lipase activity in the pooled duodenal juice was measured during the first 4 months in samples stored at −80 °C. After 6 months, however, approximately 75% of the initial activity remained. None of the added protease inhibitors seemed to protect the lipase from degradation. On the contrary, the addition of Pefabloc to the duodenal juice resulted in a rapid decrease in the lipase activity (data not shown).

We have previously performed in vitro digestion assays using HGJ and HDJ aspirated from only one individual [16, 28–30]. During five aspiration periods, this individual produced HGJ with pepsin activities ranging from 12 to 95 U/ml (average 59±34 U/ml) and HDJ with total proteolytic activities ranging from 19 to 27 U/ml (average 23±4 U/ml). Due to the intra- and intersubject variability observed and in order to have enough juice to perform several comparable in vitro digestion studies, aspirated

Table 2 Individual differences in the composition of human gastric and duodenal juice

Volunteer	Total protein in HGJ (mg/ml)	Pepsin (U/ml)	Total protein in HDJ (mg/ml)	Total proteolytic (U/ml)	Amylase (U/ml)	Bile acids (mM)	Bilirubin (μM)
1	nd	nd	nd	21.0	nd	nd	nd
2	nd	70.5	nd	16.0	nd	nd	nd
3	nd	20.6	nd	16.4	nd	nd	nd
4	nd	7.4	nd	14.4	nd	nd	nd
5	nd	nd	nd	19.6	nd	nd	nd
6	nd	7.3	nd	16.6	nd	nd	nd
7	1.0	32.4	2.0	13.0	24.9	3.2	47.5
8	1.0	55.9	1.0	12.0	49.8	0.9	22.5
9	1.3	59.5	2.4	19.8	35.5	3.6	57.1
10	0.8	nd	1.3	11.7	0.0	1.3	5.5
11	1.3	nd	2.6	25.4	33.0	3.4	47.3
12	0.9	nd	1.4	11.5	22.0	3.3	69.0
13	1.4	nd	1.1	23.9	52.6	4.2	85.2
14	1.5	nd	0.5	10.4	29.2	2.2	39.8
15	0.9	nd	1.4	5.6	10.0	3.3	58.0
16	1.1	42.2	2.2	24.7	25.8	4.5	32.8
17	1.7	45.2	0.6	22.8	28.6	1.6	232.6
18	0.5	32.0	1.0	9.4	13.0	1.0	17.9
Average	1.1	37.3	1.4	16.3	27.0	2.7	59.6
SD	0.3	21.5	0.7	5.8	15.2	1.3	58.9

Pepsin activity (n=10) and total protein concentration (n=12) in individual human gastric juices. Total proteolytic activity (n=18), amylase activity, bile acid, protein and bilirubin concentrations (n=12) in individual human duodenal juices

HGJ human gastric juice, *HDJ* human duodenal juice, *nd* not determined, *SD* standard deviation

gastric and duodenal juices collected from 18 individuals were pooled. A total of 1,300 ml HGJ and 2,100 ml HDJ were collected. Table 3 shows the characteristics of the pooled gastric juice (18p HGJ) and the pooled duodenal juice (18p HDJ) with respect to pH, volume, enzyme activities, bile acid, total protein and bilirubin concentrations. The pooled gastric juice had a pH of 1.7, a total protein concentration of 1.2 mg/ml and a pepsin activity of 26.7 U/ml. The pooled duodenal juice had a pH of 7.0, a total proteolytic activity of 14.8 U/ml, a lipase activity of 951.0 U/ml and an amylase activity of 26.8 U/ml. The duodenal juice bile acid concentration was 4.5 mM, and the total protein and bilirubin concentrations were 2.0 mg/ml and 67.5 μM, respectively.

The stability of the enzymatic activities in the pooled gastric and duodenal juices (n=18) were further studied for a 12-month storage period at −20 °C and −80 °C (Fig. 3). The individual juices had been stored for 7 to 15 months prior to pooling the samples. During the first 3 months after pooling, the pepsin activity decreased rapidly to about 65% of the original activity at both storage temperatures. The activity, however, remained more stable thereafter if stored at −80 °C ending at 55% of the original activity whereas storage at −20 °C resulted in only 36% of the original activity remaining after 12 months. In the pooled duodenal juice, the total proteolytic activity seemed more stable and decreased by only approximately 15% after 12 months storage at both −20 °C and −80 °C. The lipase activity, however, decreased rapidly and only about 40% of the activity remained 5 months after pooling the duodenal juice. After 12 months storage at both −20 °C and −80 °C, approximately 40% of the initial lipase activity still remained. The amylase activity remained largely stable throughout the 12-month storage period.

Discussion

Several models simulating in vivo digestion have been developed using commercial enzymes. Obviously, no model can internalize all potential variability in enzyme secretion created by hormonal, peptide and neural regulation in each individual. The amount and composition of intestinal fluids vary between individuals and according to the type and amount of food ingested [4, 31, 32]. Any sampling is therefore only representative of the sampling moment, reflecting the state in which the volunteer was in at that time. In the fasting state, secretion of intestinal fluid

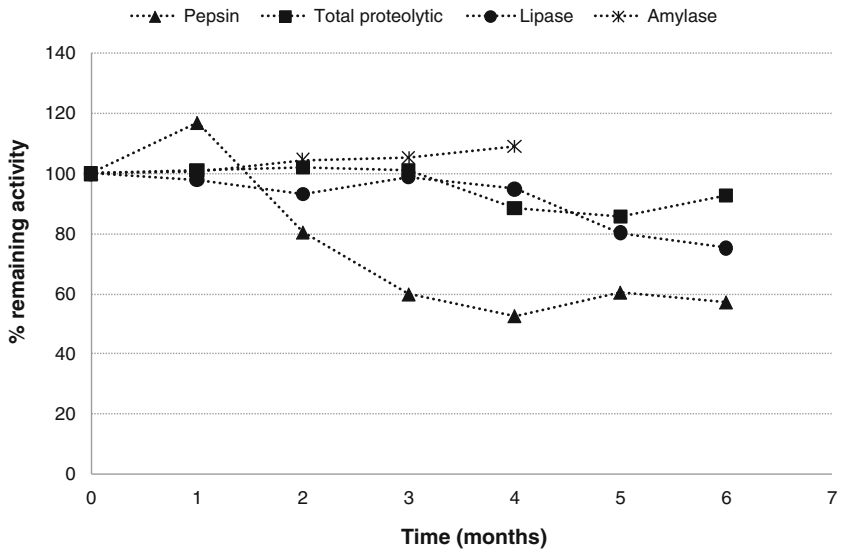

Fig. 2 Percentage remaining pepsin activity in a pooled sample of human gastric juice and remaining amylase, lipase and total proteolytic activities in a pooled sample of human duodenal juice stored at −80 °C for 6 months

is scarce [33], and collection of larger amounts requires an extended collection period. In our study, the volunteers had fasted for a minimum period of 12 h prior to collection of GI juices, so in order to stimulate pancreatic secretion, a stimulation solution was used. Test meals containing protein, carbohydrates and lipids would have enhanced the secretion but would at the same time have "contaminated" the gastric and duodenal juices intended for in vitro digestion of food components. To circumvent this problem, we used a combination of amino acids and salt known to stimulate pancreatic enzyme and bile secretion [24] during the aspiration. The volunteers were therefore neither in a fasting nor in a fed state at the time of aspiration.

The variation in composition of human gastric and duodenal juices in both fasting and fed stages has previously been characterised [31, 32]. Our study showed that the individual variations in pH, aspiration volumes, enzymatic activities and bile acids are substantial. The volunteers displayed a gastric pH ranging from 1 to 4 which is in accordance with the fasting pH of healthy young individuals reported by others to be in the range of 1.0–3.5 [34, 35]. However, this may also vary especially according to age as young children tend to have elevated gastric pH and lower amount of pepsin compared to adults [33]. Ingestion of food also temporarily raises the pH in the stomach and leads to increased secretion of pepsin [32, 36]. Our pooled HDJ had a pH of 7 while Dressman et al. [35] showed that the duodenal juice of fasting healthy young individuals had a median pH of 6.1. However, intersubject variations could be substantial [32].

The large variations in the volumes of gastric and duodenal juice observed were partly due to dilution by the stimulatory solution as shown by Holm et al. [23]. Under similar conditions, the average dilution of infusates was 1:1.64 and gastric reflux was less than 5% [23]. In addition, the emotional states of the volunteers during the aspiration could also change the secretions [37]. We observed that the more relaxed volunteers would generally produce more juice and that individuals being very uncomfortable in the situation were more likely to produce less. Furthermore, the mere thought of food seemed to increase the juice output. However, no correlation between the production volumes of gastric and duodenal juices was observed. Approximately 24-ml gastric juice is, according to Lydon et al. [38], present in the fasting stomach. We measured an average total volume of 87 ml after 2 h aspiration which is in agreement with the reported flow rate of 0.9 ml/min [33]; however, considerable intersubject variation (2 to 355 ml) was observed. Daily

Table 3 Characterisation of the pooled gastric juice sample (18p HGJ) and the pooled duodenal juice sample (18p HDJ) from 18 volunteers

	pH	Volume (ml)	Total protein (mg/ml)	Pepsin activity (U/ml)	Total proteolytic activity (U/ml)	Lipase activity (U/ml)	Amylase activity (U/ml)	Bile acids (mM)	Bilirubin (μM)
18p HGJ	1.7	1,300	1.2	26.7	–	–	–	–	–
18p HDJ	7.0	2,100	2.0	–	14.8	951.0	26.8	4.5	67.5

18p HGJ pooled human gastric juice from 18 individuals, *18p HDJ* pooled human duodenal juice from 18 individuals

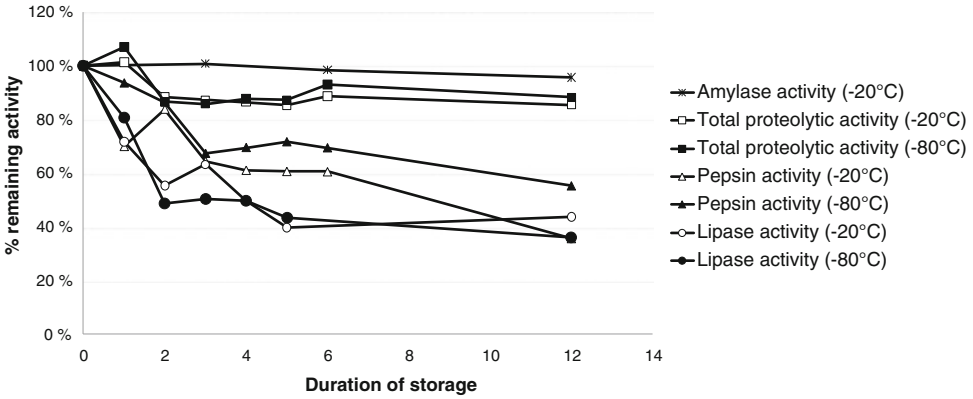

Fig. 3 Long-term storage stability of pepsin in a frozen sample of pooled human gastric juice and stability of total proteolytic, amylase and lipase activities in a frozen sample of pooled human duodenal juices stored at −20 °C or −80 °C. The individual juices had been collected and stored at −80 °C from 7 to 15 months prior to pooling them and were then observed for 12 months

outputs of duodenal juice have previously been reported to be in the range of 700–2,500 ml [39] which is in accordance with the 158-ml average volume within the 2-h collection period in this study. The duodenal juice volumes were highly variable ranging from 10 to 312 ml. This is in accordance with Moreno et al. [40] who reported that duodenal juice volumes collected over a 2-h period ranged from 40 to 355 ml.

In order to standardise the composition of the juices with the purpose of performing in vitro model digestion of food, we pooled the GI juice samples from all volunteers. The pH in the gastric juice was 1.7, and the pH of the duodenal juice was 7.0 which correlated well with the aforementioned normal pH values observed by others [32, 34, 35, 40]. Basal pepsin output has previously been reported to be approximately 0.87 mg/ml pepsin [33, 41]. The pooled gastric juice (n=18) had a pepsin activity of 27 U/ml with an individual variation of 7 to 70 U/ml. According to Holm et al. [42], average basal total proteolytic activity in duodenal juice is approximately 45 U/ml (ranging from 11 to 135 U/ml). Our pooled duodenal juice, however, displayed a total proteolytic activity of 15 U/ml with less intersubject variation (5 to 25 U/ml). The total bile acid concentration in the pooled duodenal juice (n=18) was 4.5 mM with an individual variation from 0.9 to 4.5 mM in 12 of the 18 subjects. This is in agreement with the values reported by Moreno et al. [40] and Clarysse et al. [31] showing fasting state bile acid concentration in duodenal juice ranging from 0.6 to 5.1 and 0.3–9.6 mM, respectively. Lindahl et al. [43] measured a mean protein concentration of 1.8 ranging from 0.7 to 3.9 mg/ml in the fasting gastric juice of 24 individuals. This agrees with the present study measuring a total protein concentration in the pooled gastric juice to be 1.2 mg/ml (from 0.5 to 1.7 mg/ml). The total protein concentration in the pooled duodenal juice was measured to be 2.0 mg/ml, which is somewhat lower than the 3.1-mg/ml median value observed by Kalantzi et al. [32]. We measured an amylase activity of 27 U/ml and a lipase activity of 951 U/ml in the pooled duodenal juice. Others have used different assays to measure these enzymatic activities in duodenal juice, and a comparison of the values obtained is therefore hampered.

Enzymatic activities in digestive juices have been known to deteriorate during storage [20–22, 44]. In order to use pooled gastroduodenal juices in in vitro digestion studies, the stability of the individual enzyme activities needs to be monitored and preferably stabilised to avoid substantial decrease in activities. Glycerol has by many been used as a cryoprotector to avoid loss of enzyme activities in stored gastric and duodenal juice [20, 44, 45]. The addition of 13% glycerol to freshly isolated gastric and duodenal juices only marginally protected the enzymes from degradation compared to the control in this study. This is contrary to the results by Sivakumaran et al. [44] demonstrating no loss in pepsin activity when adding glycerol in the range of 20 to 280 ml/l. In our study, the pepsin activity in the pooled gastric juice (n=18) was reduced to approximately 40–55% remaining activity after 12 months frozen storage. Some of this loss may be due to the ambient pH 1.7 in the pooled juice as the critical lowest pH of 1.8 has been demonstrated to avoid loss in pepsin activity [20]. We were, however, not able to preserve the pepsin activity by increasing the gastric juice pH to 4. Gastric juice should therefore not be stored for more than 1–2 months before being used for protein digestion to avoid loss in pepsin activity.

In the frozen pooled duodenal juice stored for 12 months, only the amylase activity remained stable. This is in agreement with the study by Muller and Ghale [21]. We observed that about 80–90% of the original total proteolytic activity remained in the pooled duodenal juice. Kelly et al. [22] showed that whereas the chymotrypsin activity remained rather stable in duodenal juice stored for 56 days, the trypsin activity displayed intersubject variability ranging from 0% to 25% decrease in activity. In the study by Muller and Ghale [21], the trypsin activity decreased to less than 60% in a third of the samples. This could indicate that a loss in trypsin activity could account for some of the loss in the total proteolytic activity observed in this study. The lipase activity appeared to remain stable up to 4 months after aspiration but then decreased steadily during storage. A

significant loss in lipase activity upon frozen storage was also observed by Kelly et al. [22] who concluded that the loss in lipase activity was caused by chymotryptic digestion as the lipase activity remained stable if the duodenal juice was incubated with a chymotrypsin inhibitor. This is also in agreement with Thiruvengadam and DiMagno [46] who confirmed this and also reported that tryptic activity had little or no effect on lipase degradation. After adding several protease inhibitors (all inhibitors of both chymotrypsin and trypsin) to freshly aspirated duodenal juice, we did, however, not observe any protection of the lipase activity during frozen storage. The addition of casein, acting as an alternative substrate for the chymotrypsin, could also have been used to protect the lipase from degradation [22]. However, as we intend to use the duodenal juice for in vitro digestion of milk proteins, the addition of any protease inhibitor or foreign protein would be out of the question.

Several models simulating in vivo digestion have been established [8, 47] including both simple static models and more sophisticated dynamic models such as the TNO gastrointestinal tract model (TIM) [48] and the dynamic gastric model and small intestinal simulation developed at the Institute of Food Research (Norwich, UK) [8]. All of these models have so far used simulated gastrointestinal fluids with commercial enzymes of animal origin and commercial bile salts. The use of human GI juices may present several advantages. The complex composition of these physiological digestive fluids better reflects the in vivo ratio of the different enzymes and isoenzymes as well as other minor components. Previous results demonstrating that human gastric and duodenal juices produce different digestion products compared to porcine purified digestion enzymes strongly support this view [16]. The inter- and intrasubject variability should always be taken into consideration. A range of concentrations of HGJ and HDJ could possibly be used to reflect the individual variability in the amounts of enzymes and bile salts as observed in this study and by others [43]. A simulation of fasted and fed situations could also be obtained by varying the amount of juices added.

Conclusions

Characterisation of individual gastrointestinal juices gives a valuable overview of the large variation in the composition, pH and volumes that can be valid during digestion and is an important tool when designing simulated digestion of foods. In order to enable the comparison of results from several digestion studies, large pooled samples of individual digestive juices have to be used. Since both pepsin and lipase activities were reduced during frozen storage, even when preservatives were added, fresh juices or juices frozen for only a short period (1–2 months) is to be preferred.

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Conflict of Interest The authors report no conflict of interest and are alone responsible for the content and writing of this manuscript.

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Paper II

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Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes

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The objective of the present study was twofold: first to compare the degradation patterns of caprine whey proteins digested with either human digestive juices (gastric or duodenal) or commercial porcine enzymes (pepsin or pancreatic enzymes) and second to observe the effect of gastric pH on digestion. An *in vitro* two-step assay was performed at 37°C to simulate digestion in the stomach (pH 2, 4 or 6) and the duodenum (pH 8). The whey proteins were degraded more efficiently by porcine pepsin than by human gastric juice at all pH values. Irrespective of the enzyme source, gastric digestion at pH 2 followed by duodenal digestion resulted in the most efficient degradation. Lactoferrin, serum albumin and the Ig heavy chains were highly degraded with less than 6 % remaining after digestion. About 15, 56 and 50 % Ig light chains, β-lactoglobulin (β-LG) and α-lactalbumin remained intact, respectively, when digested with porcine enzymes compared with 25, 74 and 81 % with human digestive juices. For comparison, purified bovine β-LG was digested and the peptide profiles obtained were compared with those of the caprine β-LG in the digested whey. The bovine β-LG seemed to be more extensively cleaved than the caprine β-LG in the whey. Commercial enzymes appear to digest whey proteins more efficiently compared with human digestive juices when used at similar enzyme activities. This could lead to conflicting results when comparing human *in vivo* protein digestion with digestion using purified enzymes of non-human species. Consequently the use of human digestive juices might be preferred.

Human gastric juice: Human duodenal juice: Commercial porcine enzymes: Whey proteins: β-Lactoglobulin

In vivo digestion of proteins is a complex process depending on several factors such as (a) the amount and composition of the digestive enzymes, (b) the pH, (c) the transit time in the various parts of the gastrointestinal tract and (d) the overall composition of the food ingested[1–3].

Human digestion of milk and milk-derived products may release peptides of different sizes available for paracellular or transcellular uptake and thus enter the systemic circulation and exert biological effects[4,5]. Several bioactive peptides have in the recent years been identified after *in vitro* enzymic digestion of milk proteins. Most of these studies have applied commercial digestive enzymes often of porcine origin[6–10]. Although commercial porcine enzymes provide an easy-to-use option to study *in vitro* digestion, their validity in mimicking physiological digestion might be questioned. Human digestive juices consist of a variety of enzymes, inhibitors and bile salts that collectively contribute to the digestion of macromolecules[11,12].

In vitro gastric digestion assays have frequently been performed at pH 1–2[3,8,10,13]. However, gastric pH varies from 1·0 to 3·5 in the fasting state of healthy adult individuals[14] and increases to a median of 6·7 during a meal[15]. The type of food ingested[2] will also influence the gastric pH. In cases with short transit times (liquid food) or with high buffering capacity of the food, the pH may never decrease to the levels used in most assays. Furthermore, patients suffering from gastro-oesophageal reflux disease are frequently treated with proton pump inhibitors (PPI) leading to steady-state gastric pH values of > 4 for 24 h to diminish oesophageal injury[16]. The activity of the gastric proteases (pepsins) is highly influenced by pH, with decreased activity above pH 4·5[12]. Low activity of the pepsins delays gastric protein digestion, resulting in an increased amount of intact protein reaching the intestine. During duodenal digestion, the pH is normally about 6·2–6·7[3,17]; however, pH values up to 7–8 are recorded both in *in vivo* and *in vitro* studies[18,19].

We investigated whether digestion of caprine whey proteins would produce bioactive peptides. To do this, an *in vitro* method that would mimic the events taking place during

Abbreviations: IgHC, Ig heavy chains; IgLC, Ig light chains; α-LA, α-lactalbumin; LF, lactoferrin; β-LG, β-lactoglobulin; SA, serum albumin; WPCG, caprine whey protein concentrate.

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British Journal of Nutrition

human gastrointestinal digestion was needed. Consequently, the aim of the present study was to compare the degradation of caprine whey proteins digested with either human digestive juices or with commercial porcine digestive enzymes. In addition, the effect of different gastric pH values, 2, 4 or 6, on the gastroduodenal degradation of the proteins was studied.

Whey proteins consist primarily of β-lactoglobulin (β-LG), α-lactalbumin (α-LA), lactoferrin (LF), serum albumin (SA) and immunoglobulins. The main protein is β-LG, making up about 50–60 % of the protein fraction of both caprine and bovine whey[20,21]. Whey proteins are considered high-quality proteins due to their elevated content of essential amino acids and are used to increase muscle gain in athletes[22] as well as for the elderly[23]. Moreover, whey proteins are rich sources of bioactive peptides[24–27]. The β-LG peptide produced during the *in vitro* digestion of caprine whey proteins was compared with bovine β-LG peptides obtained with the same enzymes at gastric pH 2.

Materials and methods

Materials

Whey protein concentrate from goats' milk (caprine whey protein concentrate; WPCG) was produced from sweet, cheese whey with a protein content of 81 %[28]. Bovine β-LG was purchased from Arla Foods (95 %, variant A + B; Arla Foods, Videbæk, Denmark; PSDI-2400). Pepsin A (77 163, 655 U/mg) was isolated from porcine stomach mucosa (Fluka BioChemika, Buchs, Switzerland) and Corolase PP (Ch 6946; 350 proteolyticU/mg) derived from pig pancreas glands, a mixture of trypsin, chymotrypsin amylase, lipase and several amino- and carboxypeptidases (Röhm GmbH, Darmstadt, Germany), was used. Hb from bovine blood was provided by Sigma (St Louis, MO, USA) and Hammarstein casein was purchased from Merck Co. (Darmstadt, Germany). All reagents used for HPLC and MS were of HPLC grade.

Aspiration of human gastric and duodenal juices

To follow up and extend previous work on *in vitro* digestion of caprine whey[28–30] the gastric and duodenal juices employed were obtained from the same individual (healthy male, no medication). The pepsin and total proteolytic activities of these juices were within the normal range observed in twelve individuals (men and women, preliminary results). The proteolytic enzymes were obtained in the activated state by collecting human gastric and duodenal juices according to Holm *et al.*[31]. In brief, a three-lumen tube (Maxter Catheters, Marseille, France) enabled both simultaneous instillation of stimulation solution in the duodenum, and aspiration of gastric and duodenal juices. The stimulation solution (70 g/l sucrose, 1·8 g/l NaCl, 3·2 g/l L-phenylalanine and 2·3 g/l L-valine in water) was instilled close to the papilla of Vater (100 ml/h) to stimulate the production of pancreatic enzymes and the human duodenal juices was aspirated some 18 cm distally. Aspirates were collected on ice, centrifuged (4500 ***g*** for 10 min) to remove mucous and cell debris before being frozen in samples and stored at −20°C before use.

Enzymic activities of gastric and duodenal enzymes

Porcine pepsin A and human gastric juices were analysed for pepsin activity at pH 3·0 with Hb as substrate as described by Sánchez-Chiang *et al.*[32]. Corolase PP and human duodenal juices were analysed for total proteolytic activity at pH 8·0 with casein as substrate, as described by Krogdahl & Holm[33]. In brief, the enzymes were incubated with substrate for 10 min at 37°C and the reactions were stopped by the addition of TCA. After an overnight sedimentation at 4°C the samples were centrifuged for 10 min at 3000 ***g*** and 4°C. The enzymic activities were measured as the difference in absorbance at 280 nm of the TCA soluble fractions. One unit of enzyme activity (U) was defined as the amount (ml or mg) of enzyme giving a difference in absorbance of 1·0 at 280 nm in 10 min at 37°C. The enzyme assays were run in triplicate and performed three or more times.

In vitro *digestion of caprine whey proteins and bovine β-lactoglobulin*

A two-step *in vitro* protein digestion assay (Fig. 1) was performed in duplicate at 37°C to simulate digestion in the stomach and duodenum as described by Almaas *et al.*[28]. Either human digestive juices or porcine enzymes were used at similar enzymic activities. In the first step, simulating gastric digestion, the pH was adjusted to 2, 4 or 6 with 2 M-HCl and 5 U of porcine pepsin A or human gastric juices was added per g protein in the solution. The second step simulating duodenal digestion was performed by adjustment to pH 8 with 2 M-NaOH adding 16 U of either Corolase PP or human duodenal juices per g protein in the solution. Both steps lasted 30 min and samples were extracted at the start, during step 1 (at 10, 20 and 30 min) and during step 2 (at 15 and 30 min). Samples were transferred directly on ice and frozen within 5 min to stop the enzymic reactions. For comparison, bovine β-LG was digested in duplicate only at gastric pH 2 at the same conditions as described above.

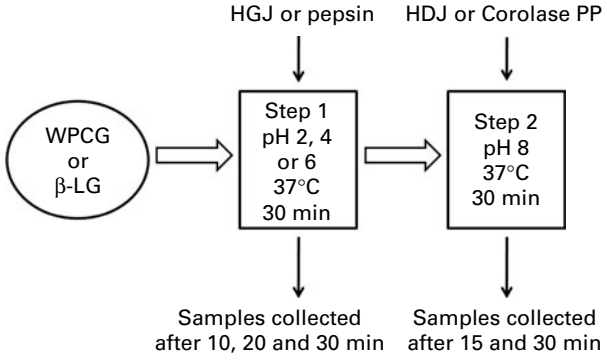


Fig. 1. Illustration of the *in vitro* digestion assay performed in two steps using either human or porcine gastrointestinal enzymes[28]. Whey protein concentrate from caprine milk (WPCG) or bovine β-lactoglobulin (β-LG) were digested at 37°C in two 30 min steps to simulate the digestion in the stomach and the duodenum. Samples were extracted 10, 20 and 30 min after the addition of human gastric juice (HGJ) or porcine pepsin, and after 15 and 30 min following the addition of human duodenal juice (HDJ) or Corolase PP. Step 1 was perfomed at pH 2, 4 or 6 for WPCG, and at pH 2 for β-LG.

Visualisation of protein degradation by SDS-PAGE and ImageQuantTL analysis

The degradation profiles of the whey proteins were assayed by SDS-PAGE using the Bio-Rad Mini-PROTEAN® 3 Cell system (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK). Each sample was run at least four times. Standard Laemmli reagents[34] and protocols were used to visualise protein degradation on 15 % polyacrylamide separating gels. Hydrolysates were mixed with 2 × SDS sample buffer (0·125 M-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl, pH 6·8, 4 % SDS, 20 % glycerol, 2 % 2-mercaptoethanol, 0·03 mM-bromphenol blue) and boiled for 5 min before application to the gel. A low-molecular-weight marker (LMW-SDS Marker Kit; GE Healthcare, Little Chalfont, Bucks, UK) was used. Electrophoresis was performed at constant 200 V for 50 min and Coomassie Brilliant Blue R-250 was used to visualise the separated proteins. To evaluate the degree of protein degradation, the ImageQuantTL 7.0 software (GE Healthcare Bio-Sciences, Uppsala, Sweden) was used. The individual protein bands in the undigested WPCG were set to 100 % and the percentage remaining was calculated based on the average of at least four gels.

In-gel digestion of caprine whey protein concentrate proteins

Whey proteins were separated on a 15 % SDS-PAGE gel. Each band (1–6; Fig. 2(a)) was excised out of the gel, and subjected to in-gel reduction, alkylation, and tryptic digestion[35]. In brief, 10 mM-dithiothreitol in 100 mM-ammonium bicarbonate (Ambic) was added to reduce the cystines (56°C; 45 min). Cysteine alkylation was carried out in the dark in a solution of 55 mM-iodoacetamide in 100 mM-Ambic (room temperature; 30 min). In-gel digestion was performed using 6 ng/μl trypsin (V511A; Promega Corp., Madison, WI, USA) in 50 mM-Ambic containing 5 mM-$CaCl_2$ (37°C, overnight). Before the mass spectrometer analyses, the trypsinated proteins were desalted and concentrated using C18 ZipTips (OMIX; Varian, Inc., Palo Alto, CA, USA) according to the manufacturer's instructions. Elution was performed with 2 μl 0·1 % formic acid in 60 % acetonitrile and diluted in 8 μl 0·1 % formic acid.

Prefractionation of digested proteins using size exclusion chromatography

Size exclusion chromatography was used to improve the separation of peptides and remove undigested proteins in the hydrolysates before mass spectrometric analyses. Freeze-dried samples of digested caprine whey proteins and bovine β-LG were dissolved to a concentration of 4 mg/ml (w/v) in a 100 mM-sodium acetate buffer mixed containing 30 % (v/v) acetonitrile and 0·1 % (v/v) trifluoroacetic acid at pH 5·5 (mobile phase). The samples were sterile filtered (0·22 μm Millex® GP filter, Millipore Express® polyethersulfone (PES) membrane; Millipore Corp., Billerica, MA, USA) and samples of 50 μl were added at a flow rate of 0·5 ml/min. Fractionation was carried out on a Tricorn™ Superdex Peptide 10/300 GL gel filtration column (GE Healthcare) with a linear separation range from 100 to 7000 Da. Both duplicates from the *in vitro* digestion assays were run seven or more times and the resulting peaks were collected and freeze dried.

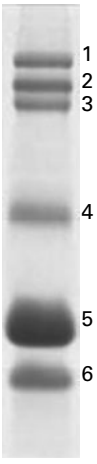
Desalting and concentration of peptides

Freeze-dried peptide fractions (peaks) prepared by size exclusion chromatography were dissolved in 0·1 % (v/v) formic acid. The samples were desalted and concentrated using self-made columns consisting of C18 column material (3M Empore C18 extraction disks; 3M Bioanalytical Technologies, St Paul, MN, USA) inserted into Eppendorf GELoader micropipette tips (Eppendorf, Hamburg, Germany). The peptides were eluted using 2 μl 70 % acetonitrile–0·1 % formic acid (v/v).

Nano-LC MS of peptides

Desalted and concentrated mixtures of peptides were diluted in 10 μl 1 % (v/v) formic acid before they were loaded onto a nanoACQUITY™ Ultra Performance LC® (Waters Corp.,

(a)



(b)

Band protein	Species	Accession no.	Sequence coverage†	Score
1 Lactoferrin	*Capra hircus*	gi88702503	21 %	721
2 Serum albumin	*Ovis aries**	gi57164373	34 %	895
3 Immunoglobulin heavy chains	*Capra hircus*	gi147744654	34 %	137
4 Immunoglobulin light chains	*Capra hircus*	gi61378762	19 %	204
5 β-Lactoglobulin	*Capra hircus*	gi125912	48 %	574
6 α-Lactalbumin	*Capra hircus*	gi125998	19 %	141

Fig. 2. Proteins identified in the caprine whey protein concentrate. (a) Proteins separated as individual bands by SDS-PAGE. (b) Identification of the protein band by nano-LC MS/MS. * All identified peptides were also present in serum albumin from *Capra hircus*; the mascot software did, however, not list this species. † Percentage of protein sequence covered by the identified peptides.

Milford, MA, USA), containing a 3 μm Symmetry® C18 Trap column (180 μm × 22 mm; Waters) in front of a 3 μm Atlantis™ C18 analytical column (100 μm × 100 mm; Waters). Peptides were separated with a gradient of 5–90 % (v/v) acetonitrile, 0·1 % (v/v) formic acid, with a flow of 0·4 μl/min eluted to a Q-TOF Ultima Global mass spectrometer (Micromass/Waters) and subjected to data-dependent tandem MS analysis. Peak lists were generated by the ProteinLynx Global server software (version 2.1), and the resulting pkl files were searched against the National Center for Biotechnology Information (NCBI) non-redundant protein sequence databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass tolerances used in the search were 100 parts per million (ppm), and fragment mass tolerance was 0·1 Da. The taxonomy used in the search was mammalia. Data were acquired over a mass:charge ratio of 400–1500 Da, detecting peptides with two or three charges. Thus, only peptides with mass above 800 and below 4500 Da were subjected to collision-induced fragmentation and further processing.

Statistical analysis

Statistical evaluation of the effect of enzyme source (porcine or human) and pH (2, 4 and 6) on protein degradation was performed on four or more SDS-PAGE gel runs per sample using Minitab 15 (Minitab Inc., State College, PA, USA). The experiment was designed as a 2 × 3 unbalanced factorial design, and a two-way ANOVA (general linear model) was used to analyse the results with Tukey's test for pairwise comparisons. The percentage remaining of each intact protein band after 10, 20 and 30 min gastric digestion or after 45 or 60 min gastric and duodenal digestion was compared at the $P<0·05$ level. Bartlett's and Levene's tests were used to test for equal variances after transforming the data by the square root. The tests found unequal variances only for the means of the Ig heavy chains (IgHC). Nevertheless, these results were included in the further analysis.

Results

Identification of proteins in the caprine whey protein concentrate

The major proteins in the caprine whey as visualised by SDS-PAGE were identified by in-gel trypsination followed by nano-LC MS/MS analysis (Fig. 2). Bands 1–6 were identified as the following proteins (molecular weight in decreasing order): LF, SA, IgHC, Ig light chains (IgLC), β-LG and α-LA.

Effect of pH and enzyme source on the digestion of whey proteins

The amounts (%) of intact whey proteins remaining after gastric and duodenal digestion are shown in Table 1. Gastric pH had a significant effect on the proteolysis of all the proteins ($P<0·001$) except for β-LG ($P=0·385$), with pH 2 resulting in the most extensive degradation. Furthermore, based on the amount of intact proteins, the porcine pepsin was overall more efficient than the human gastric juice at degrading the whey proteins. Digestion at gastric pH 4 followed by duodenal digestion resulted in lower degradation of all the proteins with both porcine and human enzymes. However, the porcine pepsin and pancreatic enzymes seemed to be more efficient than the human digestive juices. At gastric pH 6, very low degradation of all proteins was observed for both types of gastric enzymes (approximately 80–100 % intact protein), with the consequence of reduced duodenal digestion.

The degradation profiles of the digested whey proteins at gastric pH 2 are shown in Fig. 3. Fig. 3 illustrates differences in the degradation of the minor proteins LF, SA and Ig, and the main proteins, β-LG and α-LA, in whey by human and porcine enzymes. Porcine pepsin A digested the LF, SA and IgHC and IgLC efficiently, leaving only 2, 5, 6 and 28 % intact proteins, respectively. In comparison, significantly more intact protein was detected ($P<0·0001$) when human gastric juice was used, with 19, 32, 72 and 67 % of the LF, SA and IgHC and IgLC remaining, respectively. However, digestion with human gastric and duodenal juices resulted in a rapid degradation of the LF, SA, IgHC and IgLC (1, 3, 5 and 25 % intact protein).

Table 1. Amount of intact protein remaining (%) after digestion of caprine whey proteins with either human digestive juices or porcine enzymes

(Mean values and standard deviations)

	Gastric digestion†				Gastric and duodenal digestion‡			
	HGJ		Pepsin A		HGJ and HDJ		Pepsin A and CPP	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
LF								
pH 2	19	7	2**	1	1	2	2	1
pH 4	61	9	50	5	21	6	31	5
pH 6	96	13	81	13	64	16	60	12
SA								
pH 2	32	8	5**	5	3	2	2	2
pH 4	69	17	25**	7	29	9	16*	4
pH 6	102	15	92	11	97	19	88	24
IgHC								
pH 2	72	7	6**	3	5	2	2	1
pH 4	108	18	78**	14	79	22	62	10
pH 6	114	20	99	18	94	23	90	25
IgLC								
pH 2	67	14	28**	10	25	6	15	7
pH 4	111	21	74**	8	63	13	54	23
pH 6	110	9	104	11	53	13	44	9
β-LG								
pH 2	112	20	90*	14	74	9	56*	11
pH 4	116	11	97*	11	89	12	89	12
pH 6	105	14	103	13	99	13	100	15
α-LA								
pH 2	105	8	66**	9	81	10	50**	16
pH 4	115	14	94*	9	120	15	96	8
pH 6	108	15	99	8	111	16	104	20

HGJ, human gastric juice; HDJ, human duodenal juice; CPP, Corolase PP; LF, lactoferrin; SA, serum albumin; IgHC, Ig heavy chains; IgLC, Ig light chains; β-LG, β-lactoglobulin; α-LA, α-lactalbumin.

Mean value was significantly different from that when using human digestive juices at the same pH: * $P<0·05$, ** $P<0·01$.

† Gastric digestion was with either HGJ or porcine pepsin A performed at pH 2, 4 or 6. Average numbers are based on four SDS-PAGE gels or more.

‡ Gastric digestion was followed by duodenal digestion at pH 8 using either HDJ or porcine pancreatic enzymes (CPP). Average numbers are based on four SDS-PAGE gels or more.

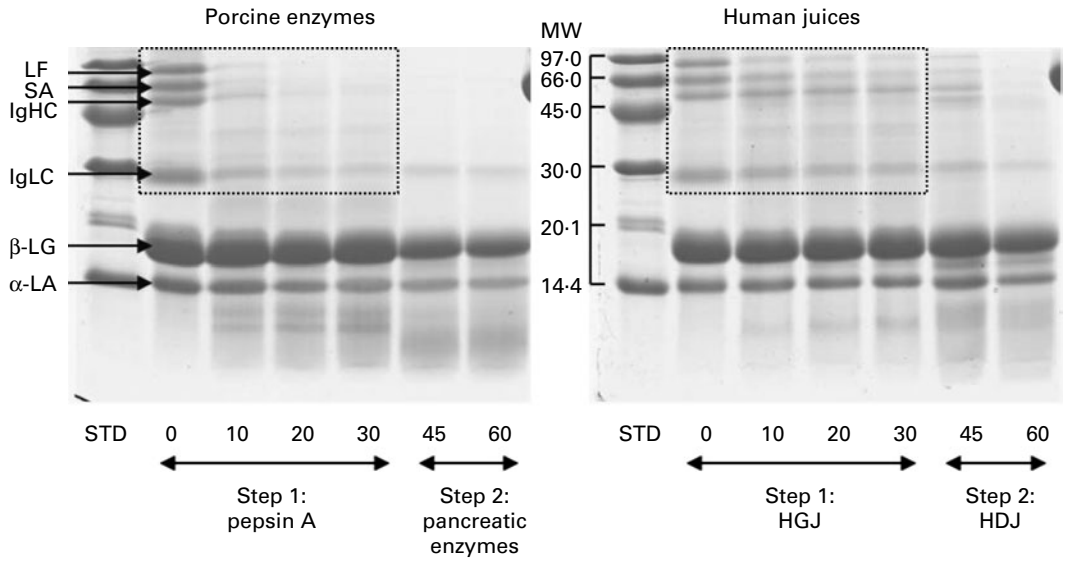


Fig. 3. SDS-PAGE (15 %) protein profiles of caprine whey protein concentrate (WPCG) digested with either human gastroduodenal juices or porcine digestive enzymes. Step 1: gastric digestion at pH 2. Step 2: duodenal digestion at pH 8. In both gels: lane 1, low-molecular-weight (LMW) marker; lane 2, undigested WPCG; lanes 3–5, WPCG digested with gastric enzymes (pepsin A or human gastric juice (HGJ)) for 10, 20 and 30 min; lanes 6 and 7, WPCG digested with gastric enzymes for 30 min followed by duodenal enzymes (porcine pancreatic enzymes or human duodenal juice (HDJ)) for 15 min (total time 45 min) and 30 min (total time 60 min). The molecular weights (MW) of the standards (STD) (LMW, in kDa) are marked at the side of the gel showing the digestion of WPCG with human enzymes. The framed areas show the proteins in whey where the most pronounced difference in enzyme source was observed. Arrows indicate the main bands before digestion: lactoferrin (LF); serum albumin (SA); Ig heavy chains (IgHC); Ig light chains (IgLC); β-lactoglobulin (β-LG); α-lactalbumin (α-LA).

The main whey proteins, β-LG and α-LA, seemed very resistant to gastric degradation with human gastric juices, whereas the porcine pepsin was significantly more efficient, leaving 90 % β-LG intact ($P=0{\cdot}0253$) and 66 % α-LA intact ($P<0{\cdot}0001$). The following duodenal digestion with the porcine pancreatic enzymes resulted in 56 and 50 % protein remaining, respectively, compared with a significantly higher amount observed with human gastroduodenal enzymes, 74 % β-LG ($P=0{\cdot}0285$) and 81 % α-LA ($P=0{\cdot}0003$).

Special attention was paid to the degradation of LF, an antibacterial protein in whey, with time (Fig. 4). Within the 30 min gastric digestion at pH 2, the porcine pepsin A was significantly more efficient ($P<0{\cdot}0001$) compared with human gastric juice and only 6 % intact LF remained after 10 min. Human gastric juices resulted in a more gradual degradation, with approximately 36 % LF remaining after 10 min and 19 % remaining after 30 min. Gastric pH 4 resulted in a markedly delayed degradation, and the digestion was overall very low at gastric pH 6. The difference in the amount of LF remaining after duodenal digestion with either porcine or human enzymes was not significant ($P>0{\cdot}05$).

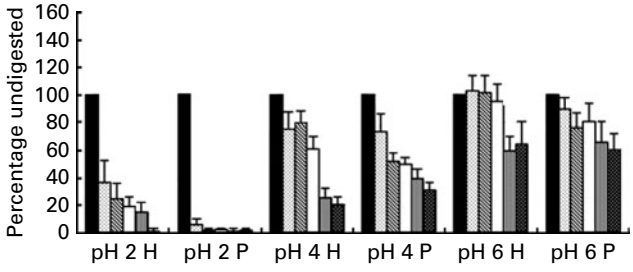


Fig. 4. Degradation profiles of lactoferrin after digestion of caprine whey protein concentrate (WPCG) with either human (H) or porcine (P) enzymes. Samples were extracted after simulated gastric digestion for 10, 20 and 30 min at pH 2, 4 or 6 followed by simulated duodenal digestion for 15 and 30 min. (■), 0 min; (▨), 10 min; (▧), 20 min; (□), 30 min; (▥), 45 min; (▩), 60 min. Values are means, with standard deviations represented by vertical bars. Significant differences ($P<0{\cdot}0001$) were obtained only at pH 2 for WPCG digested with human (pH 2 H) compared with porcine enzymes (pH 2 P).

Peptides identified after in vitro *gastroduodenal digestion*

Nano-LC MS/MS was used to identify the peptides produced by *in vitro* digestion after separating out peptides in the range of 100–7000 Da using gel filtration. Peptides from all the major proteins in the caprine whey including fragments of β-casein and κ-casein glycomacropeptide were identified (data not shown). Fig. 5 highlights the origin of peptides within the β-LG primary sequence when caprine whey was digested with human or porcine gastrointestinal enzymes using a gastric pH of 2. Two replicates were run and the peptides identified in both replicates were included in the tabulation. An overview of the β-LG peptides identified after digestion of caprine whey using human and porcine enzymes (gastric pH 2, 4 and 6) are given in Supplemental Appendix 1A and B, respectively. Bovine β-LG was digested at gastric pH 2 as a comparison with the digested β-LG in the caprine whey, and the peptides produced were identified. The sequence coverage was above 70 % and almost identical areas in the β-LG primary sequence were covered irrespective of the enzyme source as seen in Fig. 6. More peptides were found when pure bovine β-LG was digested compared with the β-LG in the digested caprine whey (Supplemental Appendix 1C).

Discussion

There are many reports in the literature on the physiological function of bioactive peptides produced by *in vitro* digestion

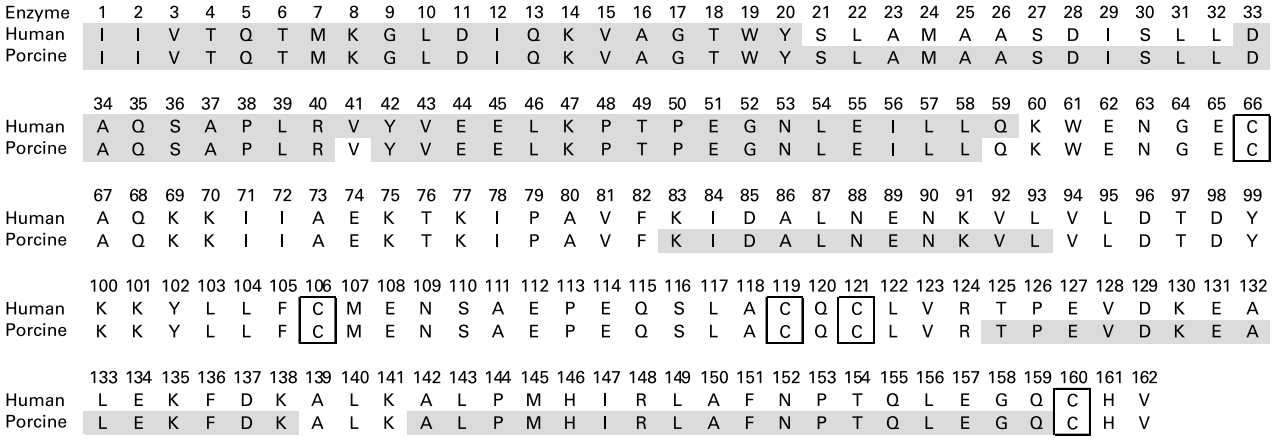


Fig. 5. Primary structure of caprine β-lactoglobulin. Grey sections denote the location of peptides identified by MS/MS after a two-step *in vitro* digestion assay. Step 1: gastric digestion at pH 2. Step 2: duodenal digestion at pH 8. Disulfide bridges are located between Cys66 and Cys160 and between Cys106 and Cys119/121.

of whey proteins using commercial enzymes from animal sources. There are, however, relatively few comparable data on digestion using human gastrointestinal enzymes. Since gastric pH seems to vary considerably in humans according to age, diet, health condition, etc, three different pH values, 2, 4 and 6, representing common variations in gastric pH were included in the present study.

As a general trend, the presented results showed that commercial porcine enzymes digested the caprine whey proteins more rapidly than the human digestive juices used at similar enzymic activities. Especially during the gastric digestion all the whey proteins seemed to be more rapidly degraded by the porcine pepsin than by human gastric juice irrespective of gastric pH. Human gastric juice contains a combination of different pepsin isoforms. Pepsin A (3a, b, c and 1) constitutes about 90 %, whereas 10 % of the proteases in non-stimulated human gastric juice is found to be pepsin C (gastricsin)[36]. The additional pepsin isoforms present in the human gastric juice may be less efficient at degrading the caprine whey proteins than porcine pepsin A alone or their activities may have different pH-optima.

Furthermore, Tang *et al.*[37] demonstrated how the proteolytic rate of gastricsin and pepsin depends on the substrate used. To determine the enzymic activity of the human gastric juices and porcine pepsin A, the substrate employed was Hb. When using a complex substrate such as whey the various pepsin isoforms may have different affinities for whey proteins as compared with their affinity for Hb.

In general, we observed that the lower the pH during the gastric digestion, the more degraded were the proteins in both steps. At gastric pH 2 all the proteins were significantly more degraded with porcine pepsin compared with human gastric juices ($P<0{\cdot}05$) especially for the minor whey proteins. Further degradation with porcine pancreatic enzymes or human duodenal juices showed only minor difference in the amount of intact protein remaining. Gastric digestion at pH 4 resulted in a significantly lower degradation compared with pH 2 for LF, SA and the immunoglobulins ($P<0{\cdot}05$). At pH 6 most of the proteins resisted the gastric digestion and the duodenal digestion was even more delayed.

The most resistant protein seemed to be β-LG and α-LA which were hardly degraded during gastric digestion irrespective of pH. This is in agreement with Chatterton *et al.*[38], who showed that α-LA and β-LG in bovine milk digested with human neonatal gastric juice were more resistant to

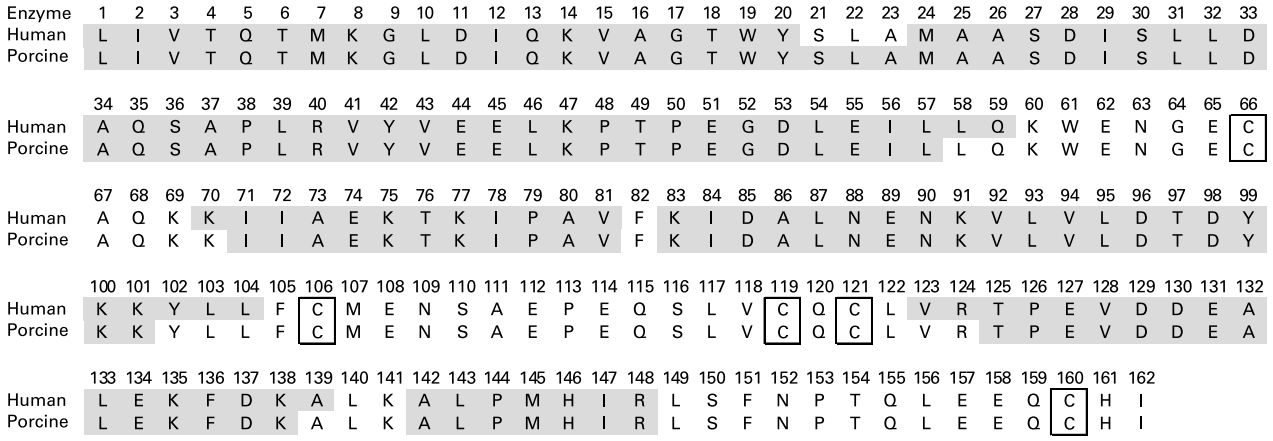


Fig. 6. Primary structure of bovine β-lactoglobulin. Grey sections denote the location of peptides identified by MS/MS after a two-step *in vitro* digestion assay. Step 1: gastric digestion at pH 2. Step 2: duodenal digestion at pH 8. Disulfide bridges are located between Cys66 and Cys160 and between Cys106 and Cys119/121.

British Journal of Nutrition

degradation at pH above 4 compared with pH 2. Schmidt *et al.*[39] also showed a marked decrease in peptic degradation of α-LA at pH 4 compared with pH 2 and 3, whereas β-LG was hardly degraded with pepsin at all pH values. However, as the whey proteins are digested, the high-molecular-weight proteins such as LF and SA may be cleaved to peptide fragments that may add to the bands of the smaller proteins when running SDS-PAGE. This could make the quantification of the degradation of the low-molecular-weight proteins (β-LG and α-LA) somewhat inaccurate, and is probably the reason why values above 100 % were recorded for these proteins during digestion (Table 1).

When studying the digestion of proteins, individual variations in gastric pH may be taken into consideration[1]. When food is ingested, the pH in the stomach tends to rise depending on the composition and quantity of the meal, but decreases gradually as HCl is secreted and the stomach empties[1,17,38]. Individuals treated for gastrointestinal reflux disease with proton pump inhibitors (PPI) have a dramatically reduced secretion of HCl, and as a result the gastric pH is kept well above 4[16,40]. At this elevated pH, degradation of normal food proteins may be significantly impaired compared with the digestion observed at low gastric pH. This pH-related resistance to hydrolysis can affect the allergenicity of food proteins as epitopes can either remain stable or be degraded[41]. Increased gastric pH could also possibly result in the production of bioactive peptides further down the gastrointestinal tract due to delayed digestion in addition to delayed release of amino acids.

The reduced gastric digestion by human gastric juices compared with porcine pepsin was to some extent compensated for by the activity of human duodenal juices (Table 1). The presence of bile salts in the duodenal juice used may also affect the proteolytic activity. Gass *et al.*[42] demonstrated that the addition of a 10 mmol/l physiological bile acid mixture significantly enhanced the digestion of β-LG and BSA by trypsin and chymotrypsin. The concentration of bile salts in the fasting state varies widely among individuals, but is usually within the range of 2·6–6·4 mM[1,17]. Postprandial duodenal bile salt concentration has been shown to peak 30 min after ingestion of a test meal[1,43], reaching a mean of 14·5 mmol/l. The human duodenal juice used in the present study was collected in a semi-fasting state (secretion stimulated by amino acids and sucrose) and therefore probably much less bile salts are present compared with duodenal juice collected after administration of a meal. However, the yellow colour of the human duodenal juices (bilirubin) indicated the presence of bile salts that in increased amounts may enhance the proteolysis compared with the porcine pancreatic enzyme mixture (Corolase PP), which, according to the manufacturer, contains only traces of bile salts.

The peptide profile of β-LG was studied in more detail since this protein is known to be rather resistant to degradation by pepsin[44,45]. The β-LG peptide profiles of the digested WPCG confirmed a more extensive degradation by porcine enzymes with a higher amount of peptides detected compared with the digestion with human enzymes.

The identified peptides released after digestion with human enzymes compared with the porcine enzymes differed to some extent. No peptides containing any of the cysteine groups after digestion of bovine β-LG was detected. Shorter peptides covering β-LG Cys160 were, on the other hand, identified in one of the replicates of the digested WPCG. Pecquet *et al.*[46] identified a peptide consisting of fragment 61–69 linked to fragment 149–162 via the disulfide bond Cys66-Cys160. The digested β-LG may contain intact cyclic peptides of various lengths linked through Cys106-Cys119/121 and/or Cys66-Cys160. These peptides were, however, not identified, indicating that either larger intact cyclic peptides or fragments too small to be detected by our method were produced.

To conclude, the present study compared the *in vitro* gastrointestinal digestion of caprine whey proteins by using either human or porcine enzymes at different gastric pH values. Differences in the degradation profiles of the individual proteins indicated that perhaps human digestive enzymes should be preferred over pure commercial enzymes from other species when mimicking human digestion. Elevated gastric pH resulted in delayed protein digestion irrespective of the enzyme source. However, further studies are needed to establish if there is a clinical significance for this finding.

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E. K. E. performed most of the experiments as well as writing the manuscript. H. H. donated his gastric and duodenal juices and assisted when performing the enzymic analyses. E. J. suggested the MS techniques to be used and helped with the interpretation of the acquired Mascot peptide files. R. A. performed the digestion of bovine β-LG, prefractionation and preparation for gel filtration. T. G. D. and M. J. participated in the research planning and interpretation of data. G. V. coordinated the research and designed the experiments. All authors contributed during the research planning, discussion of the results as well as during the revision of the manuscript and have approved the final version.

The authors report no conflict of interest.

Supplemental Appendix 1 is available online only at http://journals.cambridge.org/action/displayJournal?jid=bjn

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Paper III

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Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice

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Abstract

Peptides in caprine whey were identified after *in vitro* digestion with human gastrointestinal enzymes in order to determine their antibacterial effect. The digestion was performed in two continuing steps using human gastric juice (pH 2·5) and human duodenal juice (pH 8) at 37°C. After digestion the hydrolysate was fractionated and 106 peptides were identified. From these results, twenty-two peptides, located in the protein molecules, were synthesised and antibacterial activity examined. Strong activity of the hydrolysates was detected against *Escherichia coli* K12, *Bacillus cereus* RT INF01 and *Listeria monocytogenes*, less activity against *Staphylococcus aureus* ATCC 25 923 and no effect on *Lactobacillus rhamnosus* GG. The pure peptides showed less antibacterial effect than the hydrolysates. When comparing the peptide sequences from human gastrointestinal enzymes with previously identified peptides from non-human enzymes, only two peptides, β-lactoglobulin f(92–100) and β-casein f(191–205) matched. No peptides corresponded to the antibacterial caprine lactoferricin f(14–42) or lactoferrampin C f(268–284). Human gastrointestinal enzymes seem to be more complex and have different cleavage points in their protein chains compared with purified non-human enzymes. Multiple sequence alignment of nineteen peptides showed proline-rich sequences, neighbouring leucines, resulting in a consensus sequence LTPVPELK. In such a way proline and leucine may restrict further proteolytic processing. The present study showed that human gastrointestinal enzymes generated different peptides from caprine whey compared with non-human enzymes and a stronger antibacterial effect of the hydrolysates than the pure peptides was shown. Antimicrobial activity against pathogens but not against probiotics indicate a possible host-protective activity of whey.

Key words: Antibacterial peptides: Caprine milk proteins: Human gastrointestinal enzymes

During recent years milk proteins have been recognised as a valuable source of bioactive peptides, demonstrating various health benefits in humans. The content of these proteins may vary between different species[1,2]. Many of the derived peptides display antibacterial activity against a broad spectrum of bacterial strains, both Gram-positive and Gram-negative[3–5]. These milk peptides are mainly characterised by low molecular weight (MW), an increased number of ionic groups and an exposure of hydrophobic groups[6]. All the naturally occurring whey proteins, such as β-lactoglobulin (β-LG), α-lactalbumin (α-LA), immunoglobulins, lactoperoxidase, lysozyme (LZ) and lactoferrin (LF), including the glycomacropeptides in cheese whey, have been reported to be the source of bioactive peptides when digested enzymically[7–9].

It is well known that LF, LZ, lactoperoxidase and immunoglobulins possess properties that inhibit bacterial growth, as part of the natural host defence system in humans protecting against a great number of pathogenic micro-organisms[10–12]. Fragments of β-LG prepared with commercial enzymes such as alcalase, pepsin or trypsin, produce peptides that inhibit several types of bacteria, both Gram-positive and Gram-negative[7,13]. Other studies have shown that digestion of α-LA with pepsin, trypsin or chymosin release antimicrobial peptides[14]. In addition, glycomacropeptide has received much attention due to its ability to attach to enterotoxins from various bacteria; for example kappacin, a monophosphorylated sequence, has been reported to possess antibacterial activity against *Streptococcus mutans* and *Escherichia coli*[8].

Abbreviations: ATCC, American Type Culture Collection; FA, formic acid; α-LA, α-lactalbumin; LF, lactoferrin; β-LG, β-lactoglobulin; LGG, *Lactobacillus rhamnosus* GG; LZ, lysozyme; MW, molecular weight; OD, optical density; WPCG, caprine whey protein concentrate.

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British Journal of Nutrition

The most studied antibacterial components in milk and whey are probably LF and LZ. Both generate peptides during enzymic hydrolysis and the peptides possess strong inhibitory effects against various bacteria. Mine *et al.*[15] identified two antibacterial peptides produced from LZ, by pepsin and subsequent tryptic digestion, which demonstrated strong inhibitory effects against *Staphylococcus aureus* and *E. coli*. During the last decade, research on peptides derived from LF has received increased attention since the derivatives strongly inhibit both Gram-positive and Gram-negative bacteria[16–18]. Several of these antimicrobial peptides have been sequenced and synthesised, including bovine lactoferricin f(17–41) and lactoferrampin f(268–284)[19,20]. These peptides showed a broad antibacterial effect against strains of *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella enterica* and *Listeria monocytogenes*[21]. On the other hand, it has also been reported that LF can increase the growth of bacteria such as probiotic strains of *Lactobacillus*[22].

Although most of the research has been performed with bovine milk, similar results have also been observed for human, ovine, murine, equine, donkey and caprine milk. One of these peptides, lactoferricin C, has been identified as caprine lactoferrin f(14–42)[23]. This peptide showed strong antimicrobial activity against various types of bacteria[24,25]. In most previous studies commercial proteolytic enzymes from animal or plant origin were used[3,18,26]. The questions therefore arise whether these peptides are released during human gastrointestinal digestion and in what quantity are they generated. Finally, the physiological relevance in humans remains to be proved.

Only a few human studies have been performed that could confirm the many *in vitro* studies using proteolytic enzymes. Human ingestion of an LF solution (1·5 %) showed that only 20 % of holo- and 38 % apo-LF was digested in the stomach[27]. Another digestion study with milk and yoghurt as test meals showed that very few fragments derived from whey proteins were released during digestion[28]. We have previously shown that digestion of whey proteins is very dependent on the gastric pH. At pH 2, a significantly higher degradation of whey proteins was observed by human gastrointestinal enzymes as compared with at pH 4[29]. Since pH in the human stomach seems to vary with age and buffering capacity of the diet, the peptides generated may also vary between individuals, leading to highly variable physiological effects. It has been shown previously that β-LG, α-LA and LZ in bovine and caprine milk are very resistant to digestion with human gastrointestinal enzymes[30] and peptides generated from these proteins will probably be present in a rather low concentrations. Digestion of milk proteins from other species may be different, as β-LG from equine milk[30] was rapidly degraded by human gastrointestinal juices.

Human gastrointestinal enzymes are a complex mixture of proteases, amylases and lipases that exist in different isoforms in combination with inhibitors, bile salts, bilirubin and other minor components that may all influence protein degradation[31,32]. In a study performed with β-LG the degradation profile was very different after the addition of bile salts[33]. Consequently, purified commercial enzymes from animal or plant origin and human digestion juices seem to generate different peptides from caprine whey[29,34]. Peptides available to the intestinal brush-border surface after digestion may be structurally different and display different physiological effects.

The objective of the present study was, first, to examine whether antibacterial peptides were produced from caprine whey after human gastrointestinal digestion and, second, to compare the peptides obtained with previously identified peptides using purified non-human enzymes.

Materials and methods

Whey protein concentrate from caprine milk

Caprine milk was collected from the university farm, and caprine whey protein concentrate (WPCG) with about 81 % (w/v) protein was produced by rennet precipitation and ultrafiltration at the university pilot plant[34]. WPCG is denoted as sample A in the antibacterial screening results.

Aspiration and human gastrointestinal enzymes

Human proteolytic enzymes were obtained according to Almaas *et al.*[34] and Holm *et al.*[35]. The present study was carried out to follow up and extend our previous studies on *in vitro* digestion of caprine milk and whey. The gastric and duodenal juices were obtained from the same individual as previously described (healthy male, no medical treatment) consisting of pepsin and total proteolytic activities that are close to the mean value observed in eighteen individuals (men and women; EK Ulleberg, I Comi, H Holm, EB Heggset, M Jacobsen and GE Vegarud, unpublished results)[29]. In brief, aspiration was performed by a three-lumen tube that enabled simultaneous instillation of saline in the duodenum and aspiration of gastric and duodenal juice. Saline (100 ml/h) was instilled close to the papilla of Vater and duodenal juice aspirated some 18 cm distally. The juice was immediately cooled down and frozen at − 20°C. Aspirates were collected several times during a period of 6 months. Before further use the aspirated samples of gastric and duodenal juice were pooled into two separate batches to avoid variations in enzyme activity. The aspirate containing the gastric juice was characterised by pH and pepsin activity (U/ml) and the duodenal juice by pH and total proteolytic activitity (U/ml). Pepsin activity in the human gastric juice was assayed with Hb as the substrate[36]. Total proteolytic activity in the human duodenal juice was assayed with casein as the substrate[37]. A unit of enzyme activity (1 U) is defined as the amount of enzyme that produces an absorbance reading of optical density (OD) 1·0 at 280 nm in 20 min at 37°C. More than three parallels of the enzyme assays were used.

In vitro *model digestion*

A modified *in vitro* digestibility assay (AOAC official method 982.30)[38] was performed in two steps, using human gastric juice and human duodenal juice according to Almaas *et al.*[34].

A protein sample of 10 ml 5 % (w/v) WPCG (81 % protein) was acidified to pH 2·5 with 2 M-HCl, and incubated with 50 μl (0·4 U) human gastric juice for 30 min at 37°C. pH was adjusted to pH 7–8 with 1 M-NaOH, and 400 μl (13 U) human duodenal juice was added during continuous stirring for 30 min at 37°C. Samples were redrawn during the digestion, put on ice, frozen and then freeze-dried. The hydrolysate generated from the first step of digestion with human gastric juice was denoted sample B, while the hydrolysate obtained from the second step of degradation with both human gastric and duodenal juices was denoted sample C. The digestion was performed more than three times.

Separation of protein fractions by size membrane filtration

Fraction B from human gastric juice and fraction C from human duodenal juice digestion were separated in various subfractions using membranes with cut-offs at 5 and 8 kDa.

Fractions B and C were both prepared as 5 % solutions (50 g/l). The samples were filtered tangentially through a membrane with a cut-off of 8 kDa (Pellicon 2; Millipore, Billerica, MA, USA). Fraction C < 8 kDa was further separated by size filtration on a membrane with a cut-off of 5 kDa (Mini Ultra Omega SC membrane; Pall Corp., Port Washington, NY, USA). The filtrations were performed with a Masterflex pump (Millipore) and tubings (Masterflex AG, Gelsenkirchen, Germany), with pressure at 0·5 bar (7·5 psi (pounds per square inch)). The subfractions were kept on ice, and three to four washings through the membranes were carried out. The subfractions were freeze-dried after filtration. An overview of the different protein fractions is given in Table 1.

Desalting and concentration of the fractions

Freeze-dried hydrolysates and subfractions were dissolved in 0·1 % (v/v) formic acid (FA). The samples were desalted and concentrated using self-made columns consisting of C18 column material (3 M Empore C18 extraction discs; 3M Bioanalytical Technologies, St Paul, MN, USA) inserted into Eppendorf GELoader micropipette tips (Hamburg, Germany). The peptides were eluted using 2 μl 70 % acetonitrile–0·1 % FA (v/v).

Identification of peptides by nano-LC–MS

Eluted peptides were diluted in 10 μl 1 % (v/v) FA before they were loaded onto a nanoAcquity™ Ultra Performance LC (Waters Corp., Milford, MA, USA), containing a 3 μm Symmetry® C18 Trap column (180 μm × 22 mm) (Waters Corp.) in front of a 3 μm Atlantis™ C18 analytical column (100 μm × 100 mm) (Waters Corp.). Peptides were separated with a gradient of 5–90 % (v/v) acetonitrile–0·1 % (v/v) FA, with a flow of 0·4 μl/min eluted to a Q-TOF Ultima Global mass spectrometer (Micromass, Waters Corp.) and subjected to data-dependent tandem MS analysis. Peak lists were generated by ProteinLynx Global server software (version 2.1; Waters Corp.), and the resulting pkl files were searched against the National Center for Biotechnology Information (NCBI) non-redundant protein sequence databases using the MASCOT search engine (http://www.matrixscience.com). Peptide mass tolerance used in the search was 100 parts per million; fragment mass tolerance was 0·1 Da. Data were acquired over a mass/charge range of 300–1500 Da, detecting peptides with two or three charges. Then twenty-two peptides were selected and synthesised by GenScript (GenScript USA Inc., Piscataway, NJ, USA) with 85 % purity (see Table 2) based on peptide sequences from β-LG, β-casein and κ-casein glycomacropeptide (Figs 1–3) identified by the LC–MS.

Analysis of identified peptides

Of the identified peptides, nineteen were chosen to include all residues detected with minimal overlap. These peptides were analysed using Clustal 2.0.12 multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignment was analysed using the multiple sequence editor[39] (http://www.jalview.org/). Default settings were used for both programs.

Bacterial strains and culture conditions

E. coli K12, *Staphylococcus aureus* American Type Culture Collection (ATCC) 25 923 and *Bacillus cereus* RT INF01 were all obtained from the department stock collection at the Norwegian University of Life Sciences (UMB;Ås, Norway). *Listeria monocytogenes*, a culture of four undefined strains and *Lactobacillus rhamnosus* GG (LGG®; ATCC 53 103) were donated by Tine BA (Oslo, Norway). The cheese starter

Table 1. Protein fractions of caprine whey protein concentrate (WPCG), prepared by digestion with human gastric juice (HGJ) for 30 min and human duodenal juice (HDJ) for 30 min at 37°C, and further separated into subfractions by size membrane filtration

Fractions	Added gastrointestinal enzymes
A	Unhydrolysed WPCG
B	WPCG digested with HGJ
B > 8 kDa	WPCG digested with HGJ
B < 8 kDa	WPCG digested with HGJ
C	WPCG digested with HGJ and HDJ
Subfraction C, MW > 8 kDa	WPCG digested with HGJ and HDJ
Subfraction C, MW 5 kDa < C < 8 kDa	WPCG digested with HGJ and HDJ
Subfraction C, MW < 5 kDa	WPCG digested with HGJ and HDJ

MW, molecular weight.

British Journal of Nutrition

Table 2. Percentage inhibition of the synthesised single peptide sequences (0·1 mg/ml), and their protein precursors, κ-casein (κ-CN), β-casein (β-CN), β-lactoglobulin (β-LG), bovine glycomacropeptide (GMP) and bovine lactoferrin (LF) on *Escherichia coli* K12, *Bacillus cereus* RT INF01 and *Listeria monocytogenes* after 10 h growth*

		Inhibition (%)		
Protein precursor	Synthetic peptide sequence	*E. coli* K12	*B. cereus* RT INF01	*L. monocytogenes*
κ-CN	106–124	8	4	6
	109–121	10	5	7
	126–133	11	5	6
	130–139	12	6	7
	141–153	7	2	No
β-CN	1–9	9	2	No
	41–51	10	2	No
	61–72	13	3	No
	81–91	6	No	No
	99–105	8	No	No
	144–151	13	No	No
	191–205	14	No	No
β-LG	1–8	6	No	No
	9–18	8	No	No
	21–32	6	No	No
	33–39	12	No	No
	43–55	5	No	No
	71–82	7	No	No
	92–100	9	No	No
	125–134	11	No	No
	139–147	0·7	No	No
	149–159	4	No	No
Bovine GMP		No	No	No
Bovine LF		8	No	No

* All samples were run in triplicate.

culture CHR CH-N01 was obtained from Christian Hansen Laboratory AS (Hørsholm, Denmark). This culture is a mixture of *Lactococcus lactis* subsp. *lactis* (1–5 %), *Lactococcus lactis* subsp. *cremoris* (70–80 %), *Lactococcus lactis* subsp. *diacetylactis* (10–20 %) and *Leuconostoc mesenteroides* subsp. *cremoris* (5–18 %).

E. coli K12 *and Listeria monocytogenes* were cultured in brain heart infusion (BHI) broth (Oxoid; 37 g/l) at pH 7·4 and 37°C. *Staphylococcus aureus* ATCC25923 and the mixed strain starter culture CH-N01 were grown at 37°C in M17-broth (42·5 g/l, pH 7·2; Merck).

Bacillus cereus RT INF01 and LGG® (ATCC 53 103) were cultured in de Man–Rogosa–Sharpe (MRS) broth (52·2 g/l, pH 5·7; Merck) at 37°C. Active growing cultures (1 %) were used for inoculation in the growth experiments.

Assay of antibacterial activity

Freeze-dried samples of WPCG and hydrolysates (fractions A, B and C) were solubilised in water and added to growing bacteria cultures. The final protein and hydrolysate concentrations varied from 0·3 to 1·2 %[34]. These concentrations were selected since 0·6 % is the concentration of whey proteins in milk[40]. The synthesised peptide (GensScript) concentration used was 0·1 mg/ml. Bacterial growth was measured by OD at 660 or 600 nm. The experiments were repeated three times for each sample.

The number of viable cells (colony-forming units) was counted on agar plates for strains of *E. coli*, *B. cereus* and *Listeria monocytogenes*. *E. coli* and *Listeria monocytogenes* were grown on BHI–agar plates (Merck; 37 g/l) at pH 7·4 and 37°C, and *B. cereus* on MRS–agar plates (Merck; 22·5 g/l) at pH 7·0 at 37°C. All plates, three parallels of each dilution – 10^{-7}, 10^{-8} and 10^{-9} – were incubated for 48 h and then counted. Each experiment was repeated three times.

Calculations and statistics

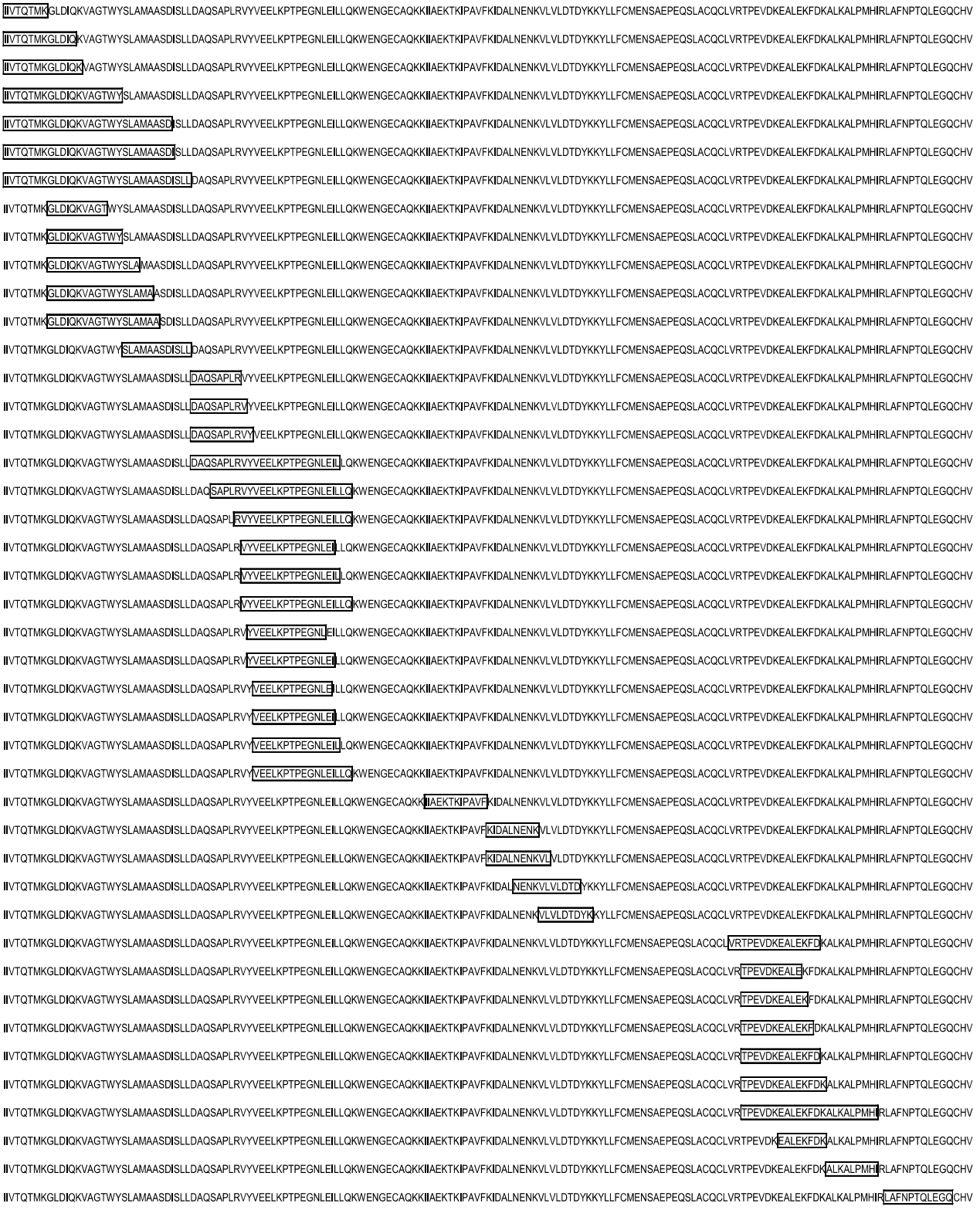
Growth inhibition was expressed as optical density ($OD_{600\,nm}$) after 10 h in comparison with the control:

$$\text{Inhibition/activation} = \left(\frac{(OD_{control,10\,h} - OD_{whey,10\,h})}{OD_{control,10\,h}}\right) \times 100\%,$$

where $OD_{control,10\,h}$ is the OD for the control bacterial curve after 10 h, and $OD_{whey,10\,h}$ is the OD for the bacterial curve with the addition of the digested whey or peptide in the growth media after 10 h.

A *t* test (two-sample, assuming unequal variances) was run to compare the different growth-curves based on data obtained after 10 h. Each experiment was repeated three times with at least three parallels, and the differences were considered significant when $P < 0{\cdot}05$. All the $OD_{600\,nm}$ measurements (recorded every 30 min) were calculated for standard deviation. The graphs are presented as mean values and standard deviations after 10 h. The rest of the standard deviation bars have been omitted for clarity in the figures.

British Journal of Nutrition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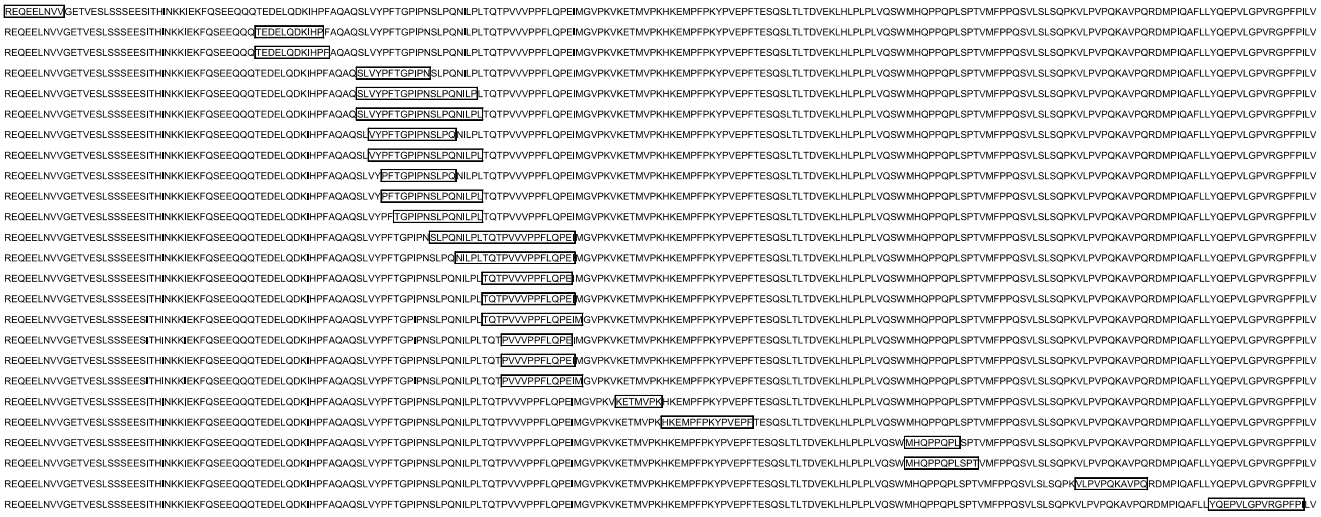

Fig. 2. Full-length amino acid sequence of β-casein and identified peptides (twenty-five framed) generated by the digestion with human gastrointestinal enzymes from human gastric juice (30 min) and human duodenal juice (30 min) at 37°C.

Results

Identified peptides generated from caprine whey proteins after in vitro *gastrointestinal digestion*

Digestion of caprine WPCG (fraction A) was carried out in two steps; first, with human gastric juice at pH 2·5 and, subsequently, with human duodenal juice at pH 7–8, resulting in hydrolysates called fractions B and C, respectively (Table 1). The hydrolysate (fraction C) was separated into subfractions according to the molecular size; high MW > 8 kDa, medium MW 5–8 kDa and low MW < 5 kDa. Peptides generated from the two-step digestion were identified by LC–MS analysis. Five peptides were identified after human gastric juice digestion, having MW 8264, 9091 and 9918 Da and of 1845 and 2190 Da. After total digestion with human gastric and duodenal juices, 106 peptides were identified originating from β-LG, β-casein derivatives of γ-caseins, κ-casein glycomacropeptide and LF. Peptides, forty-three in all, were derived from β-LG representing peptide fragments of the whole sequence of the molecule ranging from 856·4 to 3436·8 Da (Fig. 1). Fragments from β-casein, twenty-five peptides, were located mainly from the middle part of the protein (Fig. 2). The twenty-three peptides generated from the κ-casein glycomacropeptide, as a component in renneted cheese whey, were derived mainly from the N-terminal side of molecule (Fig. 3). In addition, fifteen peptides derived from LF were located in the middle of the molecule (Fig. 4). Lactoferricin f(17–42) or lactoferrampin f(268–284) were not detected, nor were any peptides originating from α-LA.

The results of multiple sequence alignment of nineteen peptide sequences from β-LG, β-casein and κ-casein glycomacropeptide are given in Fig. 5. The results showed a consensus sequence, LTPVPELK, including two prolines (P) with a valine (V) in between and neighbouring the bulky hydrophobic leucine (L). Such proline-rich sequences have been described as antimicrobial peptides[41].

Antibacterial effect of hydrolysates and peptides generated after digestion

The antibacterial effect of WPCG and the generated hydrolysates was tested in three different concentrations (0·3, 0·6 and 1·2 %). The results showed similar trends for all concentrations. Data from only 0·6 % are presented in the following part. From the growth curves of the various bacteria the growth rate and percentage inhibition were calculated (Table 3). The results obtained varied highly between the bacteria. *E. coli* K12 showed significant growth inhibition by the addition of the hydrolysate generated by gastric juice (fraction B) and an increased inhibitory effect after both human gastric and duodenal juice digestion (fraction C), as shown in Table 3. Although fraction C strongly inhibited growth (27 %), this effect seemed to be exhibited by components in the subfraction with MW > 8 kDa, since subfractions with MW < 8 kDa and < 5 kDa showed no inhibition. From the distribution of the 106 identified peptides in the molecules, twenty-two peptides were selected for synthesis and antibacterial testing. All the peptides showed a relatively moderate inhibition of *E. coli* K12 (Table 2). The peptide fragment f(191–205) derived from β-casein showed the highest antibacterial effect, with approximately 14 % inhibition. However, all peptides were less active than the hydrolysates obtained after human gastric juice and human duodenal juice digestion (fraction C), showing 27 % inhibition (Table 3). This fraction was bacteriocidal since a loss of viable *E. coli* K12 cells (measured as colony-forming units) was observed (data not shown). A clear reduction in growth rate ($\Delta OD_{600\,nm}$/h) was also shown by the same fraction C (Table 3).

After gastric digestion of whey (fraction B) no growth inhibition of *Staphylococcus aureus*, *B. subtilis*, *Listeria monocytogenes*, LGG and the cheese starter culture CHR CH-01 was observed. However, subsequent duodenal digestion (fraction C) resulted in strong activity against *Listeria monocytogenes* and *B. cereus*, with 38 and 44 % inhibition, respectively, after

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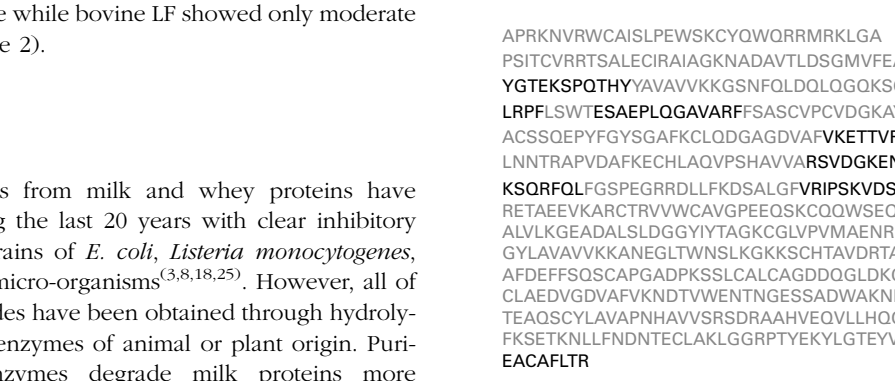

Fig. 3. Full-length amino acid sequence of κ-casein glycomacropeptide (106–169) and identified peptides (twenty-three framed) generated by the digestion with human gastrointestinal enzymes from human gastric juice (30 min) and human duodenal juice (30 min) at 37°C.

10 h growth (Table 3). For both strains it seemed to be the subfraction of high MW (MW > 8 kDa) that was most active (38 and 41 % inhibition). The lower-MW subfractions (MW < 8 kDa and MW < 5 kDa) showed less antibacterial effect, except on the cheese starter culture CHR CH-01. Only four of the twenty-two synthesised peptides showed a slight antibacterial effect (5–7 % inhibition) against *B. cereus* and *Listeria monocytogenes*. These four peptides were derived from κ-casein glycomacropeptide. All the other peptides derived from β-LG and β-casein had no inhibitory effect. The high-MW subfraction (MW > 8 kDa) of the digested whey showed high antibacterial effect; therefore, two proteins reported as antibacterial, bovine κ-casein glycomacropeptide and bovine LF (BLF), were tested. No inhibition was shown by glycomacropeptide while bovine LF showed only moderate (8 %) inhibition (Table 2).

Discussion

Antibacterial peptides from milk and whey proteins have been reported during the last 20 years with clear inhibitory effects on various strains of *E. coli*, *Listeria monocytogenes*, *B. cereus* and other micro-organisms(3,8,18,25). However, all of these bioactive peptides have been obtained through hydrolysis with commercial enzymes of animal or plant origin. Purified non-human enzymes degrade milk proteins more efficiently to shorter peptides(29,42,43). Addition of bile salt also seems to change the protein degradation of β-LG(33). The presence of other components apart from proteases seems to be important in the overall protein degradation. Human gastric and duodenal juices contain a complex mixture of proteases, amylases, lipases, inhibitors, bile salts, bilirubin and other minor components that may have an important role in the total human gastrointestinal digestion.

Proteins digested with non-human and human enzymes seem to generate different peptides both with regard to sequence and length(29). When comparing the 106 identified peptides from human enzymes with previously identified peptides from purified commercial enzymes, only two or three peptides matched. One of these peptides derived from β-LG f(92–100) has been reported earlier in both bovine and caprine species(18). Another peptide, called casecidin 15, having

Fig. 4. Full length amino acid sequence of lactoferrin (1–791) and identified peptides (fifteen in black) generated by the digestion with human gastrointestinal enzymes from human gastric juice (30 min) and human duodenal juice (30 min) at 37°C.

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2            DAQSAPLRVYVEELKPTPEGNLEILL--------
8            --------------REQEE--LNVV---------
5            -------------VLVLDT---DYK---------
15           -------------VLPVPQ---KAVPQ-------
3            ----IIA-----EKTKIPA--VF-----------
17           ---MAIPPKKDQDKTEVPA--INT----------
12           ------------KETMVPK---------------
13           -----------HKEMPFPK--YPVEPF-------
6            -----TPEVDKEALEKFDK-ALKALPMH------
11           -----NILPLTQTPVVVPP-FLQPEI--------
4            ------------KIDALNE--NKVL---------
1            --IIVTQTMKGLDIQKVAGTWYSLAMAASDISLL
14           -------------MHQPPQ---PLSPT-------
18           ------------IASAEPT--VHSTPTTE-----
10           ---SLVYPFTGPIPNSLPQ---NILP--------
16           --------YQEPVLGPVRG---PFPI---------
7            -------------LAFNPT---QLEGQ-------
19           ------------NTVDNPEASSESIA--------
9            ------------TEDELQD---KIHPF-------

Consensus                    LTPVPELK
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Fig. 5. Clustal multiple sequence alignment of nineteen peptides. Peptides no. 1–7 are derived from β-lactoglobulin, no. 8–16 from β-casein and no. 17–19 from κ-casein glycomacropeptide. The consensus sequense, LTPV-PELK, is shown with leucine (L), proline (P) and valine (L).

the sequence f(191–205) derived from caprine β-casein has been previously reported in bovine colostrum[44]. However, the reported casecidin 17 f(191–207) from κ-casein was not identified in the present study. These are surprising results, considering the many identical amino acid sequences in the caprine and bovine milk proteins.

A relatively high amount of proline seemed to be present in the nineteen peptide sequences shown by multiple sequence alignment analysis. A clustering sequence, LTPVPELK, containing two prolines with a valine and two hydrophobic leucines could constitute a possible common motif that plays a role in the proteolytic attack by human enzymes. This is in accordance with reports that proline restricts proteolytic processing[45]. Short proline-rich sequences together with hydrophobic residues such as leucine and phenylalanine have also been described as antimicrobial peptides[46].

Another observation in conflict with previously published reports was the absence of peptides identified from LF. No lactoferricin, LFcinC f(14–42), or lactoferrampin, LFampinC f(268–284), was identified in the present study even though these peptides have been reported with animal proteolytic enzymes and have also been identified in the gastrointestinal tract of mice[47,48]. However, the *in vivo* studies by Troost *et al.*[27] and Chabance *et al.*[28] showed that most of the LF was intact after gastric digestion (30 min) and only a few peptides were identified from whey proteins in milk after 30 min, 2 h and 4 h of ingestion.

Concerning the high potent antibacterial effect reported in the literature by purified peptides from milk proteins[21,25,44], a relatively low effect of peptides derived from β-LG, β-casein and κ-casein glycomacropeptide on *E. coli* K12, *B. cereus* and *Listeria monocytogenes* was shown in the present study. The hydrolysate obtained after gastrointestinal digestion of whey had a much stronger antibacterial effect than the single peptides. This might be due to either a low concentration of peptides used or that the hydrolysate contained a complex mixture of high- and low-MW proteins and peptides that may act in a synergistic manner. Surprisingly, neither the peptides nor the digested whey had any antimicrobial effect on the probiotic strain LGG; it seemed rather to be activated by the hydrolysate. This may play a role in fermented milk products such as milk and yoghurts that are on the market today.

It should be realised that the amount of peptides released from whey protein with gastrointestinal enzymes is relatively low, since 65–70% of β-LG and 90–98% of α-LA are still intact after human gastric and duodenal juice digestion.

Table 3. Percentage growth inhibition of *Escherichia coli*, *Bacillus cereus* and *Listeria monocytogenes* after 10 h (optical density (OD) at 600 nm) comparing control culture without added protein with protein fractions and subfractions

Bacterial strain	Protein fractions added to the culture	Percentage inhibition after 10 h	Growth rate† ($\Delta OD_{600\,nm}$/h)
E. coli K12	Control	–	0·8
	A: unhydrolysed WPCG	5**	0·8
	B: hydrolysate step 1 with human gastric juice	13**	0·7
	C: hydrolysate step 2 with human duodenal juice	27**	0·12
	Subfraction C MW > 8 kDa	23**	0·13
	Subfraction C MW 5–8 kDa	1**	0·8
	Subfraction C MW < 5 kDa	0**	0·8
B. cereus RT INF01	Control	–	0·34
	A: unhydrolysed WPCG	0**	0·34
	B: hydrolysate step 1 with human gastric juice	2**	0·34
	C: hydrolysate step 2 with human duodenal juice	44**	0·19
	Subfraction C MW > 8 kDa	41**	0·20
	Subfraction C MW 5–8 kDa	2*	0·34
	Subfraction C MW < 5 kDa	2**	0·34
L. monocytogenes	Control	–	0·22
	A: unhydrolysed WPCG	0**	0·22
	B: hydrolysate step 1 with human gastric juice	2**	0·22
	C: hydrolysate step 2 with human duodenal juice	38**	0·17
	Subfraction C MW > 8 kDa	38**	0·17
	Subfraction C MW 5–8 kDa	2**	0·22
	Subfraction C MW < 5 kDa	2**	0·22

WPCG, caprine whey protein concentrate; MW, molecular weight.
* $P<0·05$, ** $P<0·005$.
† Growth rate was calculated in the logarithmic growth phase between 2 and 4 h after inoculum.

BJN British Journal of Nutrition

Peptides from β-LG only were identified and no peptides from α-LA[29,30]. These results seems to be in agreement with *in vivo* studies of Chabance *et al.*[28] showing that only a few peptides from whey proteins were detected in the duodenum after human ingestion of milk or yoghurt. Questions arise why proteins such as β-LG and α-LA are more or less resistant to degradation and whether they and other polypeptides are degraded further in the jejunum or by intracellular proteases.

In conclusion, the present study showed that human gastrointestinal enzymes generate few peptides from caprine whey after gastric digestion compared with duodenal digestion. Identification of the peptides in the hydrolysates was different from previously reported peptides using purified non-human enzymes. Strong antibacterial effects were observed on *E. coli*, *B. cereus* and *Listeria monocytogenes*. Pure peptides were less inhibitory compared with the fractionated whey hydrolysates. No effect was shown on the probiotic strain LGG. Host-protective activity of whey as a digestion product is an interesting dietary aspect that might be significant for public health.

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There are no conflicts of interest to declare.

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British Journal of Nutrition

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Effect of milk proteins and their hydrolysates on *in vitro* immune responses☆

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ABSTRACT

The aim of this study was to perform a screening of various milk protein samples of both cow and goat origin to study their *in vitro* immunomodulating properties on human peripheral blood mononuclear cells (PBMC).

The protein content in the milk of the two different species varies most notably in the amount of α_{s1}-casein. A high degree of genetic polymorphism is related to the goat α_{s1}-casein genes resulting in a variable amount of total protein in the goat milk.

The milk proteins were hydrolysed using human gastric and duodenal juice or commercial pig derived enzymes to simulate *in vivo* digestion. Although different immunomodulating effects caused by various milk protein components have been observed, the mechanisms underlying these effects are not always known. In addition, most studies on the immunomodulating properties of milk protein digests have used a wide variety of commercial enzymes to simulate *in vivo* digestion. Exploring the difference in immunomodulating properties of milk protein-derived peptides produced by the aid of enzymes from human gastric secretions, compared to those produced by commercial enzymes, is a novel approach that may be of great importance. It could help to explore which peptides are actually produced during *in vivo* early digestion of milk and how they influence the immune system.

Especially the whey protein concentrates from goat and cow showed a dose-dependent inhibition of human PBMC proliferation *in vitro*. This effect could neither be explained by a toxic effect on the PBMCs as shown by a standard viability test, nor by induction of apoptosis caused by the same milk protein samples. We suggest that intact or hydrolysed components in the milk protein samples affect the production of activation signals thereby inhibiting lymphocyte proliferation.

1. Introduction

Even though milk is considered highly nutritious, the consumption of milk on a world basis is decreasing (Boland et al., 2001). In contrast, the number of health-conscious individuals is increasing. These individuals are often interested in consuming food that, in addition to providing the necessary nutrients, also could reduce the risk of disease (Roberfroid, 2000). This has led to a growing awareness in the dairy industry for developing so-called functional foods. Milk and other dairy products seem to have potentially health-promoting constituents which are unique to this type of food. The result is an increase in research, attempting to discover hidden bioactivities within the structures of the milk proteins.

The composition of caseins in goat's milk is strongly influenced by the genetic polymorphism at the casein loci (Tziboula-Clark, 2003; Haenlein, 2006).

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The total content of caseins in goat's milk is somewhat lower than in cow's milk (Jenness, 1980; Miranda et al., 2004). This is primarily due to the high degree of genetic polymorphism of the α_{s1}-casein genes resulting in different concentrations of α_{s1}-casein in the goat milk ranging from 0 to 3.6 g/l (Grosclaude et al., 1987). The Norwegian goat breeds have a particularly high frequency of goats producing milk with no α_{s1}-casein, the so-called "null" type milk (Vegarud et al., 1999). In bovine milk, α_{s1}- and β-caseins are the main caseins whereas β-casein is the main casein in caprine milk. The α_{s2}-casein and κ-casein content in goat's milk is higher than in cow's milk (Jenness, 1980; Haenlein, 2006).

The content of the major whey proteins β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in goat and cow milks does not vary to the same extent as the caseins. The level of α-LA is approximately the same, whereas there is a slightly higher amount of β-LG in cows milk compared to goats milk (Park et al., 2007; Martin and Grosclaude, 1993; Miranda et al., 2004; Moatsou et al., 2005).

In addition to β-LG and α-LA, caprine and bovine milks contain serum albumin, lactoferrin, lactoperoxidase and immunoglobulins as well as proteose-peptone (Park et al., 2007).

Cow's milk frequently causes allergy in small children and goat's milk is believed to be a good replacement food. Lara-Villoslada et al. (2004) reported that when goat's milk was used as the first source of protein after a period of breastfeeding in mice, it was less allergenic than cow's milk. On the other hand, Ah-Leung et al. (2006) showed that goat's milk allergy occurred in children of higher age compared to infants of cow's milk allergy, and in addition a higher sensitivity of the casein fraction compared to the whey proteins was observed.

During the last decade various milk proteins and their enzymatically derived peptides have demonstrated modulating capacities on *in vitro* lymphocyte activation and proliferation (Cross and Gill, 2000). The modulating ability of the whey proteins and peptides also includes cytokine secretion, antibody production, granulocyte and natural killer (NK) cell activity and phagocytic activity (Gauthier et al., 2006).

Cross and Gill (1999) demonstrated a dose-dependent inhibitory effect of a modified whey protein concentrate on both T- and B-cell proliferation *in vitro*. They also observed that the same whey protein concentrate was able to suppress mitogen-induced T-cell secretion of γ-interferon (IFN-γ). In 2004, Mercier et al. (2004) demonstrated that a microfiltered whey protein isolate significantly increased *in vitro* lymphocyte proliferation. However, after digesting the whey protein isolate with trypsin and chymotrypsin, the stimulatory effect decreased. The same study also showed that by fractionating the hydrolysed whey protein isolate, some of the resulting peptide fractions stimulated lymphocyte proliferation even at reduced protein concentrations.

Individual whey protein fractions such as lactoferrin and lactoperoxidase are also reported to have suppressive effects on mitogen-activated lymphocyte proliferation (Wong et al., 1997; Mercier et al., 2004). Both proteins also inhibited the production of IFN-γ (Wong et al., 1997). However, this inhibitory effect was reduced when the two proteins were used together in a cell culture. The same study (Wong et al., 1997) also found that neither α-LA, nor bovine serum albumin (BSA) had any effect on T- and B-cell responses to mitogen activation.

Purified β-LG has been shown to induce proliferation of resting murine spleen cells, and the effect was amplified after digestion with pepsin, trypsin, chymotrypsin or pancreatin (Mahmud et al., 2004). Wong et al. (1998) also demonstrated that purified β-LG could promote *in vitro* proliferation of mouse spleen cells.

Reviews on bioactive milk-derived peptides (Meisel, 1998; Clare and Swaisgood, 2000) showed that many of the casein-derived peptides significantly stimulated lymphocyte proliferation. However, a study by Kayser and Meisel (1996) revealed that β-casomorphin-7 and β-casokinin-10 (both derived from β-casein) appeared immunostimulating at high concentrations and immunosuppressive at low concentrations. Since it is not yet known how much of a certain peptide can be released during enzymatic digestion, this could imply that there is a great amount of uncertainty related to what the *in vivo* effect of milk-derived peptides would be. There is nevertheless some evidence pointing in the direction of the actual formation of these peptides *in vivo*. Peptides such as the β-casomorphins have been identified in the juice of the small intestine of adult humans after they had consumed 1 l bovine milk (Svedberg et al., 1985).

The present study was performed as a screening of different milk protein samples to establish their possible immunomodulating properties on human peripheral blood mononuclear cells. Furthermore, the intention was to reveal whether a different effect would be induced by goat milk proteins compared to their cow milk analogues. In addition, we wanted to know whether the caseins and whey proteins together could induce a different modulating response on peripheral blood mononuclear cells compared to the whey proteins alone.

2. Materials and methods

2.1. Raw materials

Whey protein concentrate from cow's milk (WPC-C) was produced from pasteurised cheese whey at TINE (Norwegian Dairies), Norway.

Cheese whey was also the basis for the whey protein concentrate from goat's milk (WPC-G) which was produced at the Department of Chemistry, Biotechnology and Food Science, the Norwegian University of Life Sciences, UMB (Ås, Norway). Both whey protein concentrates had a final protein content of about 80% (w/w).

Freeze-dried BSA was obtained from Integen Company (NY, USA), and bovine skim milk (SK) was produced at TINE (Norwegian Dairy), Norway.

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats and from whole blood collected from healthy donors.

Pepsin from porcine stomach mucosa came from Sigma (St. Louis, MO, USA) whereas Corolase PP (CPP) from pig pancreas was delivered by Röhm GmbH (Darmstadt, Germany). CPP is a mixture of trypsin, chymotrypsin and several amino- and carboxypeptidases.

Human gastric (HGJ) and duodenal juice (HDJ) obtained from one individual were kindly donated by the Department of Nutrition, the University of Oslo, Norway (Almaas et al., 2006).

As cell culture medium complete RPMI 1640 (cRPMI) was used unless otherwise is specified: RPMI 1640 (Gibco, Invitrogen Corporation, Paisley, UK) was supplemented with L-glutamine, 45 μmol/l monothioglycerol, 18 mg/l garamycin (Schering-Plough, Brussels, Belgium), 9 ml/l non-essential amino acids (Invitrogen Corporation, USA), 9 ml/l sodium pyruvate (Invitrogen Corporation, Carlsbad, CA, USA) and 10% heat inactivated foetal calf serum (FCS), (Gibco, Invitrogen Corporation, Paisley, UK). All cell culture plates were flat bottom 24-/48- and 96-well Cell Culture Clusters from Costar (Corning, Acton, MA, USA).

All incubations of cells were performed at 37 °C in a humidified 5% CO_2 incubator.

2.2. Milk protein hydrolysis

Hydrolysis of milk proteins was carried out using either the commercial enzymes pepsin and CPP, or by using HGJ and HDJ. The reason for applying two different enzyme systems was to investigate the possibility of differences between the resulting hydrolysates causing different immunological responses.

A modified *in vitro* protein digestibility assay was performed in two steps according to Almaas et al. (2006). The first step used HGJ or pepsin (~4 mg/g protein in solution), and the second step used HDJ or CPP (~4 mg/g protein in solution) to mimic the digestion in the human stomach and duodenum. BSA and WPCs were hydrolysed at a concentration of 5% and the skim milk had a protein concentration of 3.3% (w/v). BSA was only hydrolysed by the commercial enzymes as it was only included as a negative control according to a previously published study (Wong et al., 1997).

Samples collected after the first step will be termed either HGJ or pepsin and the samples collected after the second step will be called HDJ or CPP in the following discussion.

A total of 17 different samples were freeze dried (Heto Drywinner 6–85) for further use in immunological assays.

2.3. SDS -polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was executed using Pharmacias PhastSystem™ (Pharmacia Laboratory Separation Division, Amersham Biosciences AB, Uppsala, Sweden) with the separation medium, PhastGel™ Homogenous 20 (Amersham Biosciences)

The electrophoresis was performed according to a standard protocol (Laemmli, 1970) and the gels with the separated proteins were stained with Coomassie blue using PhastSystem™ Development Technique with peptide fixation. A low molecular weight standard kit (LMW Calibration kit, Amersham Biosciences) was used as molecular mass markers.

2.4. Isolation of human peripheral blood mononuclear cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated from a buffy coat and from whole blood by Lymphoprep™ (Nycomed Pharma, Asker, Norway) density gradient centrifugation. The isolated PBMCs were adjusted to the proper cell concentration in cRPMI. Cells isolated from the buffy coat were frozen in liquid N_2 in freezing medium (cRPMI containing 20% dimethylsulphoxide (DMSO)). Freshly isolated PBMCs were used for the flow cytometric test whereas the other experiments were carried out using cells that had previously been frozen.

2.5. Viability test

100 μl 10, 5 or 2.5 mg/ml of WPC-G start, WPC-G HDJ or WPC-G CPP was added to 100 μl 10^6 cells/ml in cRPMI and incubated for up to four days. 10 μl of each sample was then mixed with 10 μl acridine orange/ethidium bromide mixture on a microscopic slide. Living (green fluorescence) and dead (orange fluorescence) cells were counted using a fluorescence microscope (Leitz Wetzlar, Germany).

2.6. In vitro lymphocyte proliferation assays

2.6.1. Mitogen-induced in vitro lymphocyte proliferation

Intact and hydrolysed milk protein samples were tested for their effects on lymphocyte proliferation. Each protein sample was diluted two-fold down to 0.125 mg/ml in cRPMI.

100 μl 10^6 cells/ml was added to each well in a 96-well cell culture plate. BSA was used as a negative control, and the positive cell control was without milk protein. Cells were stimulated with the polyclonal activators phytohemagglutinin (PHA), concanavalin A (Con A) or monoclonal anti-CD3 antibodies (anti-CD3) at optimal concentrations. 20 μl of PHA, Con A or anti-CD3 was added to the appropriate wells in triplicates before adding 100 μl of each concentration of the milk protein samples.

The plates were incubated for 72 h. 20 μl [^{3}H]-thymidine (0.1 μCi) was added to each well, for the last 24 h. The cells were harvested using a Filtermate harvester (Packard Bioscience, Groningen, The Netherlands), and [^{3}H]-thymidine incorporation into the cellular DNA was measured using TopCount NXT™ (Packard Bioscience, Groningen, The Netherlands).

2.7. In vitro generation of antigen experienced T cells

PBMCs were stimulated with anti-CD3/anti-CD28 coated Dynabeads (Invitrogen, Oslo, Norway) to activate the T cells. 1 ml 0.5×10^6 cells/ml and approximately 10 μl 1.5×10^8 beads/ml anti-CD3/anti-CD28 coated beads were added to each well in a 24-well plate. After 5 days incuba-

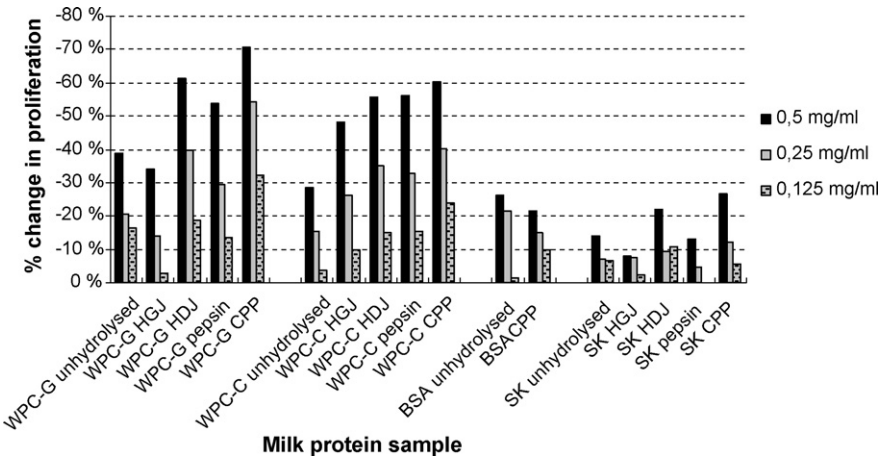

Fig. 1. Dose-dependent inhibition of anti-CD3 stimulated PBMC proliferation by different milk protein samples. Abbreviations: WPC-G: whey protein concentrate from goat milk, WPC-C: whey protein concentrate from cow's milk, BSA: bovine serum albumin, SK: skim milk, HGJ: human gastric juice, HDJ: human duodenal juice and CPP: Corolase PP.

tion, the beads were removed using a magnetic device and more cRPMI was added to sustain their growth.

2.7.1. Restimulation of antigen experienced T cells

The activators used were (1) soluble anti-CD3 at a final dilution of 1/75,000 (v/v), (2) anti-CD3 coated Dynabeads (5 beads per cell), and (3) anti-CD3/anti-CD28 coated Dynabeads (5 beads per cell) dissolved in cRPMI.

100 μl 0.5×10^6 cells/ml were added to each well in a 96-well plate. Next 20 μl of either of the three activators were added before adding 100 μl 0.5 mg/ml of each milk protein fraction in triplicates to the appropriate wells. Incubation and [^{3}H]-thymidine incorporation was as described for the mitogen-induced lymphocyte proliferation assay.

2.7.2. Flow cytometric apoptosis test

To detect whether the protein samples induced apoptosis in lymphocytes, a flow cytometric apoptosis test (APOPTEST^{TM}-FITC, Nexins Research, Kattendijke, The Netherlands) was employed.

400 μl of each milk protein sample (0.5 mg/ml) was added to 400 μl 10^6 cells/ml (freshly isolated) in a 24-well plate and incubated for 24 h. Then 200 μl of each sample (about 10^6 cells) was centrifuged for 3 min before removing the cRPMI medium from the cells. The labelling of the cells was carried out according to the manufacturer's instructions. Within 5 min after finishing the incubation, the cell samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the CellQuest^{TM} software (Becton Dickinson, San Jose, CA, USA). A total of 5000 cells were counted per sample, and the percentage of annexin V positive (apoptotic cells) versus propidium iodide (PI) positive (necrotic cells) cells was determined.

3. Results

3.1. Effect of in vitro enzymatic digestion of milk proteins

Hydrolysis of the different milk protein samples resulted in a gradual degradation illustrated by a reduced pH due to an increased amount of degradation products. In addition, the proteolysis was confirmed by SDS-PAGE clearly showing the degradation of the original protein samples after treatment with both enzyme systems. As expected, the amount of degradation products increased after treatment with HDJ or CPP when compared with the first hydrolysis step (data not shown).

3.2. Effects of milk protein samples on in vitro mitogen-induced lymphocyte proliferation

To reveal any effect on *in vitro* lymphocyte proliferation, graded amounts of milk protein preparations were added to cultures of PBMCs and stimulated with optimal concentrations of PHA, Con A and anti-CD3. Addition of the milk protein samples resulted in a dose-dependent inhibition of lymphocyte proliferation. Fig. 1 depicts data from a representative experiment carried out with anti-

Table 1
Amount of viable cells compared to the control sample after up to four days incubation. Only WPC-G hydrolysed using pepsin and CPP seemed to be cytotoxic and the effect was only detected after four days incubation. Control cells were incubated without milk protein samples

	% viable cells		
	Concentration (mg/ml)	After 24 h incubation	After four days incubation
WPC-G unhydrolysed	10	91	80
	5	97	94
	2.5	94	94
WPC-G HDJ	10	94	82
	5	92	89
	2.5	88	88
WPC-G CPP	10	88	n.d.
	5	88	89
	2.5	90	90
Control	0	87	72

WPC-G: whey protein concentrate from goat milk, HDJ: human duodenal juice and CPP: corolase PP.

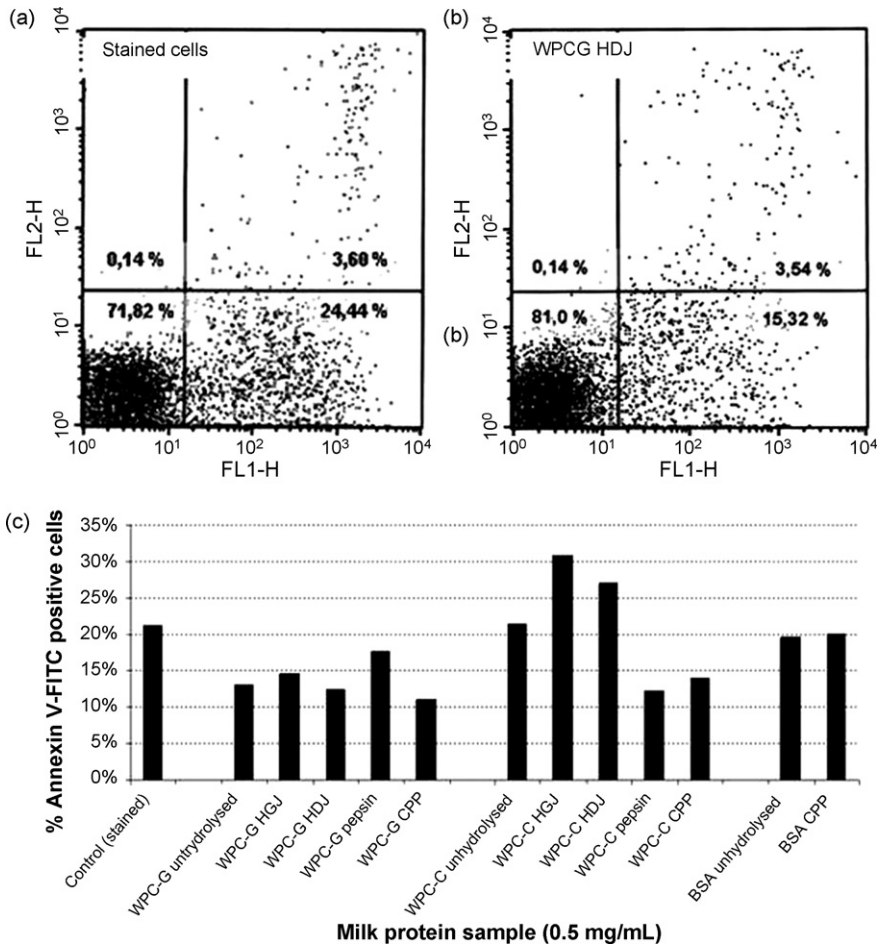

Fig. 2. Testing for annexin V/propidium iodide positivity by flow cytometry. FL1-H denotes the number of annexin V-FITC positive cells, while FL2-H denotes PI positive cells. (a) Control: annexin V-FITC and PI labeled cells without addition of WPC (b) Cells added WPC-G digested with both HGJ and HDJ. A representative picture of the distribution of annexin V-FITC and PI labeled cells added different milk protein samples. (c) Annexin V-FITC positive lymphocytes in the cell samples (average of two representative experiments). No dramatic increase in the number of annexin V-FITC positive (early sign indicative of apoptosis) cells was detected. See Fig. 1 for key to abbreviations in addition to FITC = fluorescein isothiocyanate. Control cells were incubated without milk protein samples.

CD3 stimulation. At a concentration of 0.5 mg/ml WPC-G and WPC-C significantly suppressed the lymphocyte proliferation whereas a concentration of 0.125 mg/ml had a much weaker effect. Data obtained with the other activators were similar.

BSA and skim milk preparations appeared to be less inhibitory than the other milk protein samples with only a slight suppressive effect at the highest concentration used.

The caprine WPC seemed to be somewhat more inhibitory to PBMC proliferation compared to the bovine WPC, but the major difference was, however, found between the two WPCs and the skim milk.

For all the milk protein samples, except for BSA it was evident that the most highly hydrolysed samples had the greatest inhibitory effect.

3.3. Viability test

To study whether the observed effects could be due to the protein samples being toxic to the cells, a standard acridine orange/ethidium bromide viability test was carried out. After 24 and 96 h incubation, viable and dead cells were counted in a fluorescence microscope. The results of this test are shown in Table 1.

As can be observed, WPC-G samples hydrolysed with pepsin and CPP at a concentration of 10 mg/ml seemed to have a negative effect on the number of viable lymphocytes, but only after four days of incubation. The other protein samples did not negatively affect the amount of viable cells compared to the control. Some preparations even seemed to increase cell viability. This preliminary test indicated that the milk protein samples were not toxic to the cells.

3.4. Effect of milk protein samples on apoptosis

Although we did not detect any significant toxic effect of the milk protein fractions on the cells by a standard viability test, we wanted to ascertain that the inhibition of proliferation was not due to an increased tendency to apoptosis among the cells. Testing for annexin V/propidium iodide

positivity by flow cytometry (Fig. 2) allows the discrimination between apoptotic and necrotic cells. Depicts an average of two representative experiments revealing that the milk protein samples in general did not greatly increase the number of annexin V positive cells. The number of necrotic cells (PI positive cells) was low and did not increase significantly when adding any of the milk protein samples (data not shown).

3.5. Effect of milk protein samples on in vitro T lymphocyte proliferation in the absence of accessory cells

Since the milk protein-induced inhibition of cell proliferation did not seem to be caused by a toxic effect on the cells, other options had to be checked. When cultures of PBMCs are stimulated by polyclonal activators, most responding cells are T cells. T-cell activation by mitogens and monoclonal anti-CD3 antibodies also depend upon the presence of accessory cells like dendritic cells and monocytes. Thus, an effect of milk protein samples on cell proliferation in cultures of PBMC could be due to an effect on T cells, accessory cells or both. To address this question, we first generated a population of activated T cells. After an *in vitro* expansion period these cells were for all purposes devoid of accessory cells. When restimulating such "antigen experienced" T cells with anti-CD3/anti-CD28 coated beads, the milk protein samples appeared to be slightly stimulating on T-cell proliferation. These results also demonstrated that the milk protein samples were not toxic to the cells. On the other hand, restimulation with anti-CD3 coated beads followed by the addition of the protein samples led to a decrease in [^{3}H]-thymidine incorporation and thus suppression of T lymphocyte proliferation. Restimulation of the T cells with soluble anti-CD3 antibodies which is highly dependent on accessory cells, did not induce proliferation at all. The effect was the same for WPC of both goat and cow origin.

4. Discussion

The effect of different milk protein samples on the *in vitro* proliferation of human peripheral blood mononuclear cells was investigated. Previous research has revealed both suppressive and stimulating modulatory effects on *in vitro* lymphocyte proliferation, including whole casein, α-, β- and κ-casein, whole whey protein, lactoferrin, lactoperoxidase, milk growth factor and endogenous milk immunoglobulin G (Cross and Gill, 2000).

In this study intact and hydrolysed samples of WPC-C, WPC-G, BSA and SK were investigated for their effect on *in vitro* PBMC proliferation. The proteins were hydrolysed by two sets of enzymes, either human HGJ and HDJ or commercial pepsin and CPP. No apparent difference in the immunological response induced by the two enzyme systems was observed.

A standard lymphocyte proliferation assay revealed that the incorporation of [^{3}H] thymidine was suppressed to various degrees by the different milk protein samples when stimulated with Con A, PHA and anti-CD3. The inhibition of the lymphocyte proliferation was dose-dependent for all the proteins samples, but the whey protein concentrates seemed to be more suppressive than SK and BSA. WPC from goat's milk seemed slightly more suppressive than the bovine WPC, although the major difference was found between the two WPCs and the skim milk. This could indicate that the skim milk could contain one or more immunostimulating components, such as the caseins, masking the suppressive effect of the whey proteins.

Both individual milk proteins and their enzymatically derived peptides have been demonstrated to have various effects on immune cell functions (Gill et al., 2000). The milk protein samples applied in this study consisted of a complex mixture of different proteins and peptides.

A homogenous protein or peptide fraction may be expected to have defined immunomodulating effects. However, a mixture of different fractions might not result in any modulation of immune cell functions at all. The more purified the individual milk proteins or their peptides get, the more clearly defined their immunomodulating effects would be (Cross and Gill, 2000; Wong et al., 1997). Thus, the original milk product, consisting of variable amounts of immunomodulating proteins and peptides, may possess altered immunomodulating properties compared to individual and highly purified peptides. SK for example may contain proteins/peptides that diminish or suppress the effect of the inhibitory whey protein components. For instance, research has found immunostimulating effects of caseins and casein-derived peptides (Fiat et al., 1993; Kayser and Meisel, 1996).

Both immunostimulating and immunosuppressive peptides of α_{s1}-casein origin have been observed (Gill et al., 2000). However, it is not known which of these peptides will dominate in milk after *in vivo* digestion or how they might affect each other.

Because no purification of the different milk protein constituents were performed in the present study, it is impossible to tell which of the protein components may have inflicted the suppressive effect on the lymphocytes. Instead an attempt to identify a possible mechanism for the inhibition was investigated.

The mitogen-induced lymphocyte proliferation assay is a frequently applied test system that does not distinguish between inhibition of early and late phases of the T-cell activation process.

Suppression of [^{3}H] thymidine incorporation could therefore have several possible explanations. It could either be caused by an inhibitory effect on early events in lymphocyte activation, or it could be caused by protein samples blocking the cell cycle progression at a certain stage. Other possible explanations could be that the milk protein samples could induce apoptosis or be directly toxic to the cells.

The inhibitory effect was found not to be caused by toxic effects on the lymphocytes as addition of the milk protein samples did not influence the amount of viable cells in a sensitive viability test. In addition the samples proved to have a stimulating effect on T cells when these were also stimulated with anti-CD3/ant-CD28 coated beads. This would not have been the case had the samples been toxic.

In addition, the milk protein samples did not seem to increase the amount of apoptotic cells in a culture as shown by the flow cytometric apoptosis test. This test offers a

possibility for differentiating between viable, necrotic and apoptotic cells (Vermes et al., 1995). Binding of annexin V to phosphatidylserine (PS) without the binding of propidium iodide (PI) indicates that the cell is apoptotic. Nevertheless, the binding of annexin V to PS is only indicative of an early event in the apoptotic process taking place. Whether the milk protein samples would induce apoptosis after prolonged incubation is not known.

Since the milk protein samples did not seem to induce apoptosis or be cytotoxic to the cells, other possible explanations for the inhibition of proliferation had to be investigated. Given that the majority of the cells that were activated by the activator substances were T cells, the inhibition of [^{3}H]-thymidine incorporation would most likely primarily have affected T-cell proliferation. To induce proliferation of resting, naive T cells, accessory cells expressing important costimulatory molecules are required. The inhibitory effect was therefore assumed to be caused by a direct effect on the T lymphocytes, their accessory cells or on both cell types.

As shown in Fig. 3 the milk protein samples induced significantly different modulating effects on the proliferation of antigen experienced T lymphocytes stimulated with either anti-CD3/anti-CD28-coated beads or anti-CD3 coated beads.

Whereas the proliferation of the T lymphocytes stimulated with the anti-CD3 coated beads was markedly decreased, the proliferation of T cells activated by the anti-CD3/anti-CD28 coated beads slightly increased. When the cells were stimulated with anti-CD3 coated beads the inhibition even seemed stronger than what was observed for the mitogen-activated proliferation assay. Even SK appeared to be equally inhibitory as the whey protein concentrates. The difference in the immunomodulating effect caused by the milk protein samples may be due to the fact that the anti-CD3/anti-CD28 coated beads provide different and more potent activating signals compared to anti-CD3 coated beads. When stimulating with anti-CD3/anti-CD28 coated beads both activation signal 1 and 2 are conferred to the T lymphocytes (Trickett and Kwan, 2003).

When stimulating antigen experienced T cells with anti-CD3 coated beads, the test system relies on internal activation signals to sustain proliferation. Our finding that milk protein samples inhibited anti-CD3 induced proliferation could imply that the protein samples were somehow able to block the intracellular signals in the T-cell activation process. This could further imply that the initial suppression of the PBMCs could be caused by milk protein samples affecting the signalling involved in activation of proliferation

Cross and Gill (1999) demonstrated an inhibitory effect of a modified whey protein concentrate (WPC) on both B and T lymphocyte proliferation. The same modified WPC also inhibited the production of IFN-γ in a Con A stimulated cell culture and caused a suppression in the percentage of activated T cells. This indicates that some of the components of the modified WPC alone or together were able to suppress *in vitro* lymphocyte activation. When treating the modified WPC with the digestive enzymes pepsin and pancreatin, the digested filtrate showed no immunomodulating properties against the lymphocytes. The present study on the other hand, revealed a somewhat increased immunosuppressive effect of the digested protein samples on PBMCs. This indicates that the conditions during *in vitro* digestion, as well as the enzymes applied could influence the formation of immunomodulating components.

Both inhibition and stimulation of the immune system could in a certain context be positive. By enhancing the proliferation of immune cells, potential pathogens invading the body may be defeated more quickly (Meisel and Bockelmann, 1999).

On the contrary, a suppressive effect on lymphocyte proliferation might be of great importance when ensuring a state of tolerance towards non-self sub-

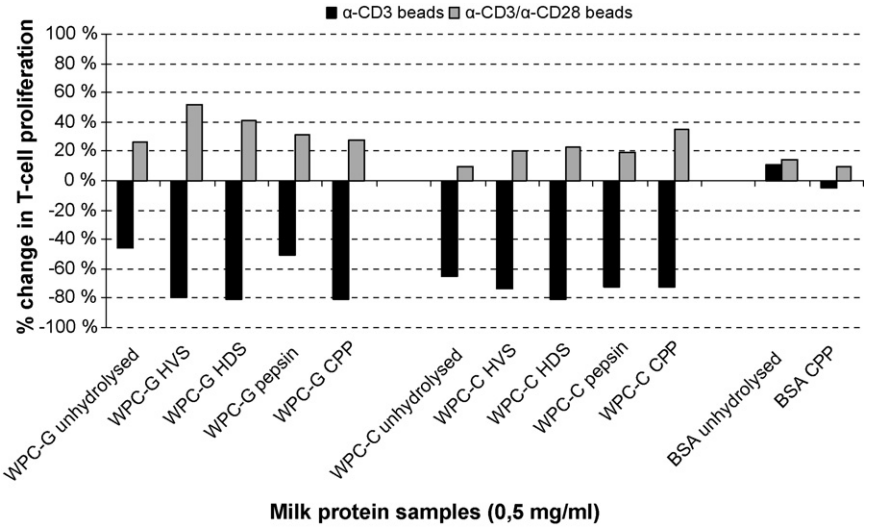

Fig. 3. Effect of whey protein samples on the proliferation of T lymphocytes restimulated using either anti-CD3/anti-CD28 coated beads or anti-CD3 coated beads. The samples seemed to augment T-cell proliferation when stimulated with the anti-CD3/anti-CD28 beads. On contrary, the stimulation by anti-CD3 coated beads in the presence of the milk protein samples seemed to inhibit lymphocyte proliferation. See Fig. 1 for key to abbreviations.

stances such as food molecules entering the body. Milk is originally intended as food for the newborn. Different milk protein-derived immunomodulating components may therefore somehow exhibit a regulatory effect on each other to avoid an unfavourable stimulation or suppression of the neonatal immune system (Cross and Gill, 2000).

In vivo studies have demonstrated that some immunomodulating peptides formed during digestion can be found in the juice of the small intestine of adult humans after consumption of bovine milk (Svedberg et al., 1985). Other studies have revealed conflicting results both between various *in vitro* assays and between *in vitro* and *in vivo* analysis of the same protein components (Gauthier et al., 2006). The review by Gauthier et al. (2006) pointed out how different raw materials and methodological approaches applied in the diverse immunological assays reviewed, could lead to conflicting results for many of the whey proteins preparation tested. There is still much left to do to confirm that results obtained *in vitro* could occur *in vivo*, however, using human digestive secretions to simulate digestion could give a better understanding of what actually occurs in the gastrointestinal tract. The peptides produced with human enzymes versus those produced by enzymes from other species could be different depending on the specificity of the enzymes.

While the majority of the *in vitro* analysis of milk-derived proteins has found immunosuppressive effects, the *in vivo* tests have revealed immunostimulating effects (Cross and Gill, 2000). It is moreover not possible to predict whether these molecules are absorbed through the epithelial cells lining the intestinal mucosa. For this reason, results from *in vitro* assays are only to be taken as indications of what may happen when potentially immunomodulating molecules are consumed. *In vitro* assays are nonetheless interesting for the identification of potential bioactive peptides in food.

Further analyses are needed to determine their relevance in the regulation of the immune response, both systemically and throughout the intestine. It is of great importance to establish if a protein preparation has the same biological function *in vivo* as it has *in vitro* before using it as an ingredient in a functional food.

5. Conclusion

The results revealed that the whey protein samples both from goat and cow showed a dose-dependent inhibition of T lymphocyte proliferation *in vitro*. This inhibitory effect is suggested to be due to interference with the generation of the important T-cell activating signals, caused by one or several milk protein-derived components. This would hinder further lymphocyte proliferation.

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Paper V

Immune cell cytokine profiles induced by different milk derived peptides

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Abstract

The aim of this study was to identify potentially immunomodulating peptides generated from caprine whey protein concentrate (WPCG) by *in vitro* digestion with human gastric and duodenal juices. To investigate for effects on the adaptive or innate parts of the immune system, both purified human T cells and dendritic cells (DCs) were studied separately. 109 peptides were identified in the WPCG digesta. Of these 22 were synthesized; ten of which were derived from β-lactoglobulin (β-LG), seven from β-casein (β-CN) and five from κ-casein glycomacropeptide (κ-CN). Proliferation of T cells restimulated with anti-CD3 coated beads was reduced by about 90% by the digested WPCG. Fractions of the digested WPCG (> 8kDa, 5-8 kDa and < 5kDA) and most of the peptides also displayed inhibitory effects, however, not to the same extent as the unfractionated sample. The WPCG hydrolysate fractions and peptides were screened for their effects on T-cell secretion of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IFN-γ and MCP-1. All peptides and fractions increased secretion of IL-1β and IL-12, while secretion of IL-4 and IL-5 was reduced by many of the peptides and by the hydrolysate fraction of low molecular weight. The peptides only affected the DC secretion of IL-6 and IL-8, whereas the levels of IL-10, IL-12 and IFN-γ were unaltered. The observed immunomodulating effects of the WPCG hydrolysate could only partly be explained by the identified peptides. Interestingly, each peptide seemed to induce unique changes in the cytokine profiles and could therefore potentially display different immunomodulating effects *in vivo*.

Keywords

Whey peptides, human gastrointestinal juices, T cells, dendritic cells, cytokines

Introduction

An increasing amount of research support that digestion of whey proteins can generate immunomodulating peptides as reviewed by Gauthier *et al*. [1] and Beaulieu *et al*. [2]. It has been suggested that immunomodulatory milk peptides may be part of regulating the development of the immune system in infants and to ease allergic reactions in atopic humans [3]. The immunomodulating effects caused by whey proteins and their hydrolysates are, however, diverse. While some milk protein components are reported to stimulate immune functions, other protein components in the same milk sample may exhibit immunosuppressive effects [4]. Unfortunately the results are sometimes contradictory possibly due to the use of different milk protein preparations, digestion models and methodologies. Data is still lacking on the characterisation of individual immunomodulating milk peptides [1]. In addition, the *in vivo* generation of these peptides and their survival through the gastrointestinal tract remains to be confirmed. *In vitro* digestion of proteins using human gastric and duodenal juices could offer a more reliable way to simulate human digestion, compared to using commercial proteases from other species [5].

Most previous research has studied the effect of whey proteins on mixed populations of leukocytes consisting of both innate immune cells such as dendritic cells (DCs) as well as lymphocytes [6-10]. In order to get one step closer to explaining the immunomodulatory role of milk peptides, it is important to study the effects on each cell type separately.

Recently, studies have been performed to reveal how the release of cytokines is affected by the presence of whey proteins and whey protein hydrolysates [8, 11, 12]. Cytokines are low molecular weight signalling molecules secreted by both immune and non-immune cells. They act by binding to specific receptors located on the producing cell, on neighboring cells or on more peripheral cells. In the gut, cytokines modulate immunity in response to internal and external stimuli [13].

The present study was based on the following aims:

1. To identify peptides released after *in vitro* digestion of a caprine whey protein concentrate by human gastric and duodenal juices
2. To compare the potential immunomodulating effect of the individual peptides with the effect of the whey protein hydrolysate
3. To study the effect of individual peptides on the secretion of cytokines by human T cells and dendritic cells separately

Materials and methods

Materials

Caprine whey protein concentrate (WPCG), with a protein content of about 81% (w/v), was produced from ultrafiltrated and spray dried whey obtained by rennet precipitation in the university pilot plant [14]. Haemoglobin (H2625) from bovine blood was provided by Sigma (St. Louis, MO) and Hammarstein casein was purchased from Merck Co. (Darmstadt, Germany). All reagents used for HPLC and mass spectrometry were of HPLC grade. Complete RPMI 1640 (cRPMI) was used as cell culture medium: RPMI 1640 (PAA Laboratories GmbH, Pasching, Austria) was supplemented with L- glutamine, 10% heat inactivated foetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria), 50 µM monothioglycerol (Sigma, Sigma, St. Louis, MO, USA), 24 µl/ml gentamycin sulphate (BioWhittaker, Walkersville, MD, USA), 1% non-essential amino acids and 1 mM sodium pyruvate (both PAA Laboratories GmbH, Pasching, Austria). All cells were maintained at 37 °C in a humidified 5% CO_2 incubator.

Aspiration of human gastric and duodenal juices

Human gastric (HGJ) and duodenal (HDJ) juices were collected according to Holm *et al.* (1988)[15] and Almaas *et al.* (2011)[16]. To follow up and extend previous work on *in vitro* digestion of caprine whey proteins [14, 17, 18], the gastric and duodenal juices were obtained from the same donor (healthy male, no medication). The pepsin and total proteolytic activities of these juices were within the normal range observed in twelve individuals (men and women, [19]). The gastrointestinal juices were collected by placing a flexible three-lumen tube that enabled simultaneous instillation of saline in the duodenum and aspiration of gastric and duodenal juice. Correct placement of the tube was monitored by radiology. Saline (100 ml/h) was instilled close to the papilla of Vater and the duodenal juice aspirated some 18 cm distally. Gastric juice was aspirated from the canalis ventriculi. Aspirates were collected on ice, centrifuged (4500 g for 10 min) to remove mucus and cell debris, frozen in aliquots and stored at -20°C prior to use.

Enzyme activities of gastric and duodenal proteases

HGJ was analysed for pepsin activity at pH 3.0 with haemoglobin as substrate according to Sanchez-Chiang *et al.* (1987) [20]. HDJ was analysed for total proteolytic activity at pH 8.0 with casein as substrate, as described by Krogdahl & Holm (1979)[21]. In brief, triplicates of human gastric or duodenal juices in three concentrations were incubated with substrate for 10 minutes at 37ºC and the reactions were stopped by the addition of trichloroacetic acid (TCA). After overnight sedimentation at 4ºC samples were centrifuged for 10 minutes at 3000 g. The enzyme activities were calculated based on the increase in absorbance at 280 nm (A_{280}) due to formation of TCA soluble peptides. Unhydrolysed proteins were used as blanks. One unit of enzyme activity (U) was defined as the amount (ml) of gastric or duodenal juice giving an increase of 1.0 in A_{280} in 10 minutes at 37°C.

In vitro digestion of caprine whey proteins

A two-step *in vitro* protein digestion was performed in a 37°C water bath to simulate digestion in the stomach and duodenum [14, 16]. A protein sample of 10 ml 5% (w/v) WPCG (81% protein) was acidified to pH 2.5 with 2 M HCl, and incubated with 50 µl (0.4 U) human gastric juice for 30 min. The pH was further adjusted to pH 7–8 with 1 M NaOH, and 400 µl (13 U) human duodenal juice was added during continuous stirring for 30 min. The digestion was performed more than three times. Samples were collected after the gastric (WPCG HGJ) and duodenal (WPCG HGJ/HDJ) steps, put on ice, frozen and then freeze-dried.

Identification of peptides by nano-LC-Q-TOF

WPCG hydrolysates from gastric and duodenal digestion were separated into fractions of varying molecular weights using membranes with cut-off at 5 and 8 kDa as described by Almaas *et al*. (2011)[16]. Three fractions were prepared; above 8 kDa, between 5 and 8 kDa and less than 5 kDa. Size exclusion chromatography was used to improve the separation of peptides and remove undigested proteins in the whey before mass spectrometric analyses [5]. The samples were desalted and concentrated by self-made C18 columns before being analysed by nano-LC-MS as described by Eriksen *et al*., 2010[5] and Almaas *et al*., 2011[16]. Of the identified peptides, 22 sequences from β-lactoglobulin (β-LG), β-casein (β-CN) and κ-casein (κ-CN) glycomacropeptide were selected and synthesised by GenScript (> 85% purity; GenScript USA Inc., Piscataway, NJ, USA).

Isolation and activation of T cells

Human peripheral blood mononuclear cells (PBMC) were isolated by Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient centrifugation from a buffy coat of a healthy donor kindly donated by Oestfold Hospital. Adherent cells, i.e. monocytes, dendritic cells and B lymphocytes, were removed by filtration through nylon wool columns (Indiveri *et al*., 1980)[22]. To ensure a homogenous T-cell population, the cells from the nylon wool columns were stimulated with anti-CD3/CD28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway). Cells were plated in a concentration of $0.5x10^6$ cells ml^{-1} and incubated with 3 beads per T cell for 5 days. Then, the beads were removed and the cell culture continued for another 4-5 days.

Restimulation of antigen experienced T cells

Hydrolysed whey protein fractions and synthetic peptides identified within these fractions were screened for their effects on the proliferation of activated T cells. Anti-CD3 coated Dynabeads or anti-CD3/CD28 coated Dynabeads (both Invitrogen Dynal, Oslo, Norway)

dissolved in cRPMI were used as activators at a concentration of five beads per cell. In a 96-well plate, 100 µl cell suspension (0.5×10^6 cells ml^{-1}) were added to each well. Next 20 µl of either of the two activators were added before adding 100 µl of undigested WPCG (0.5 mg/ml), each WPCG fraction or single peptide in triplicates to the appropriate wells. The plates were incubated for 72 h. 20 µl [^{3}H]-thymidine (0.1µCi) was added to each well, for the last 24 h. The cells were harvested using a Filtermate harvester (Packard Bioscience, Groningen, The Netherlands), and [^{3}H]-thymidine incorporation into the cellular DNA was measured using a TopCount NXT^{TM} (Packard Bioscience, Groningen, The Netherlands). The assays were performed three or more times. The results are presented as the average change in proliferation of cells incubated with whey samples divided by the control (no added sample).

Viability test

100 µl 0, 5 or 0.25 mg/ml of undigested WPCG, WPCG HGJ, WPCG HGJ/HDJ or the three fractions of WPCG HGJ/HDJ >8 kDa, 5-8 kDa and < 5 kDa was added to 100 µl 10^6 naïve T cells/ml and incubated for three days. 10 µl of each sample was mixed with 10 µl acridine orange/ethidium bromide mixture on a microscopic slide. Living (green fluorescence) and dead (orange fluorescence) cells were counted using a fluorescence microscope (LeitzWetzlar, Germany).

T cell cytokine production after incubation with milk peptides

Hydrolysed WPCG fractions and single synthetic peptides were screened for their effect on the cytokine production of activated T cells. To a 24-well plate $0.5x10^6$ cells in 0.5 ml were added per well. Next 37.5 µl anti-CD3/CD-28 coated Dynabeads were added at concentration of 3 beads per cell. The hydrolysate fractions and single peptides were then added in a concentration of 50 µg/ml and incubated for approximately 24 h at 37 °C in a humidified 5% CO_2 incubator. The experiment was performed 2-3 times per sample and the supernatants were collected and stored at -20°C prior to cytokine analysis.

The cytokine-containing supernatants were analysed using the Bio-Plex Pro assays with the Bio-Plex Cytokine Reagent Kit and the Bio-Plex Human Serum Diluent Kit (Bio-Rad Laboratories). Ten cytokines and chemokines were measured in each supernatant including interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), gamma interferon (IFN-γ) and monocyte chemoattractant protein (MCP)-1. All supernatants were diluted 1:2 in cRPMI and analysed according to the manufacturer's instructions using a fluorescence-based cytokine detection assay (Luminex-100, Bio-Rad Laboratories)

Generation of dendritic cells from monocytes

PBMCs were resuspended in a buffer of phosphate buffer saline (PBS; PAA Laboratories GmbH, Pasching, Austria) containing 0.5% bovine serum albumin and 2 mM EDTA (80 µl

per 10^7 cells). Next, CD14 Microbeads (MACS CD14 MicroBeads, Miltenyi Biotec, Sunnyvale, CA, USA) were added (20 µl per 10^7 cells) and the cells were incubated at 4°C for 15 minutes. After washing the cells in cold buffer, the cells were resuspended in 500 µl buffer. The cells were loaded onto a MACS MS column (Miltenyi Biotec, Sunnyvale, CA, USA) which was placed in a magnetic field. CD14 negative cells were washed away with buffer before removing the column from the magnetic field and eluting the CD14 positive monocytes. Monocytes were seeded at $2x10^6$ cells per well in a 24-well plate.

Immature DCs were generated from the CD14 positive monocytes by adding 25 ng/ml IL-4 and 50 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) (both from ImmunoTools GmbH, Friesoythe, Germany) to cells in fresh cRPMI on days 0 and 4.

DC cytokine production after incubation with milk peptides

The monocyte-derived DCs were incubated with hydrolysed whey protein fractions or single synthetic peptides to study their effect on cytokine production. On day 5, 900 µl fresh cRPMI with 1 µg/ml lipopolysaccharide (LPS; Sigma) was added to the cells. The hydrolysate fractions and some of the peptides (β-LG peptides f1-8, f33-40, f43-55, f71-82 and f149-159, β-CN peptides f1-9, f41-51, f81-91, f156-166 and f191-205 and κ-CN peptides f106-124, f126-133 and f141-153) were then added at a final concentration of 50 µg/ml and incubated for approximately 48 h at 37 °C in a humidified 5% CO_2 incubator. Supernatants were collected and stored at -20°C prior to the cytokine analysis.

The DC supernatants were analysed in duplicates by enzyme-linked immunosorbent assay (ELISA) for their concentrations of IFN-γ, IL-6, IL-10 and IL-12 (p70) by using R&Ds human DuoSet ELISA Development kits (R&D Systems, Abingdon, UK) and IL-8 using ImmunoTools' IL-8 matched pair for ELISA (ImmunoTools GmbH, Friesoythe, Germany). All assays were performed according to the manufacturer's instructions.

Results

Identification of peptides

The main protein in caprine whey is β-LG, making up around 50-60%. In addition, caprine whey contains α-lactalbumin (a-LA), lactoferrin (LF), serum albumin (SA) and immunoglobulins [23, 24]. Whey produced by rennet coagulation also contains κ-CN glycomacropeptide. Fragments of β-CN cleaved by the milk enzyme plasmin is also often prsent in whey especially if the milk has been refridgerated prior to renneting [25]. The digestion of a caprine whey protein concentrate by gastric and duodenal juices resulted in both intact proteins and peptides of various sizes. In total 98 peptides were identified after simulated gastric digestion. Most of these peptides were of β-CN origin (44 peptides) as shown in table 1, however, peptides were also identified from β-LG, κ-CN, SA and LF. When gastric digestion was followed by duodenal digestion, 109 peptides were identified. The majority of these peptides were of β-LG origin (43 peptides) but 23 and 25 peptides were also identified from κ –CN and β-CN respectively. In addition, some peptides were identified from α-LA, SA and LF, respectively.

From the 109 peptides identified after simulated gastroduodenal digestion, 22 peptides originating form β-LG, β-CN and κ-CN glycomacropeptide were selected to be synthesised and screened for their effects on *in vitro* cell proliferation and cytokine production (Table 2). The selected peptides cover large parts of each proteins primary sequence.

Effect of whey peptides on the proliferation of restimulated T cells

To study whether the single peptides had an effect on T-cell proliferation, antigen-experienced T cells were restimulated either by anti-CD3/CD28 or anti-CD3 coated beads in the presence of WPCG hydrolysate or single peptides.

After incubating the single peptides with T cells during restimulation by anti-CD3/CD28 coated beads, most of the peptides seemed to have little effect on proliferation. Some peptides and the hydrolysate fractions reduced the proliferation by 10-15%, while undigested WPCG and the WPCG hydrolysates inhibited proliferation by approximately 20-30% (Figure 1a).

However, when incubating the WPCG hydrolysates and whey peptides with T cells restimulated by anti-CD3 coated beads, almost all samples demonstrated suppressive effects. The WPCG digested by both HGJ and HDJ was the most potent suppressor by reducing the T-cell proliferation by 90%. As shown in figure 1b, also the undigested WPCG as well as its HGJ hydrolysate were strongly inhibitory (about 60-70% reduction). The fractions of WPCG HGJ/HDJ also displayed suppressive effects, however not to the same extent as the unfractionated sample. All κ-CN peptides as well as β-LG f92-100, f125-134 and f139-147and β-CN f41-51, f61-72 and f191-205 reduced the proliferation by approximately 20-30%. β-LG fragments f1-8 and f43-55 on the other hand, seemed to stimulate the T-cell proliferation by about 55 and 30% respectively (Figure 1b).

Effect of milk peptides on cytokine production by activated T cells

The cytokine response by T cells activated with anti-CD3/CD28 coupled beads was examined in the absence or presence of single peptides and fractions (Mw >8 kDa, 5-8 kDa and <5 kDa). Supernatants were collected after 24 hrs and the cytokines were quantified by multiplex immunoassay. The percentage change in cytokine release after addition of the various samples is shown in table 3. In general, the release of IL-1β and IL-12 was greatly increased by all the peptides, while the release of IL-4 and IL-5 was less influenced and sometimes even reduced. Secretion of IL-6, IL-8, IL-10, MCP-1 and IFN-γ was moderately stimulated by the whey peptides. Correlations were found between the release of IL-1β and IL-6 (r=0.92) and between IL-10 and IFN-γ (r=0.83). The release of IL-2 which is required for T-cell proliferation was increased by all but one sample. Two overlapping κ-CN peptides displayed quite different effects on the release of T-cell cytokines (Figure 2). The secretion of IL-2, IL-4, IL-5, IL-6 and IFN-γ was suppressed after incubation with κ-CN f106-124, whereas κ-CN f109-121increased their release. Both peptides increased the secretion of IL-1β, IL-8, IL-10, IL-12 and MCP-1.

Effect of milk peptides on cytokine production by LPS-activated DC

Fourteen peptides that influenced the secretion of T-cell cytokines were further studied for their effects on dendritic cell cytokine secretion. The secretion of five cytokines including IL-6, IL-8, IL-10, IL-12 and IFN-γ was studied, however, the peptides seemed to only affect the DC secretion of IL-6 and IL-8. Figure 3a shows that β-CN peptides f81-91 and f156-166 greatly increased the dendritic cell IL-6 secretion. A slight increase in IL-6 secretion was also observed for β-CN f41-51 whereas the other peptides and hydrolysate fractions had no effect. All the hydrolysate fractions and most of the peptides on the other hand increased the DC release of IL-8 compared to the control (Figure 3b). The peptides with only limited effect on IL-8 secretion were β-LG f33-40, f43-55 and f149-159 as well as β-CN f1-9 and f81-91 and κ-CN f141-153.

Discussion

Previous results have shown that WPCG and hydrolysates thereof decreased the proliferation of mitogen-activated PBMCs in a dose-dependent manner [18]. However, the components (intact proteins or peptides) responsible for the observed effects were not identified. In the present study the influence of single synthetic peptides and digested fractions of WPCG on proliferation of activated T cells and on cytokine release by human T cells and dendritic cells was examined. Of particular interest were the shorter peptides (approximately 800-4500 Da) as other studies have identified short milk peptide released by commercial enzymes [26, 27]. 22 of the 109 identified peptides originating from β-LG, β-CN and κ-CN in our study, were synthesised. Peptides obtained by commercial enzymes from β-LG, β-CN and κ-CN have shown multiple effects *in vitro*, acting as antihypertensive, antithrombotic, antioxidant, antimicrobial and immunomodulating agents [28-30]. Most ingested protein is digested and subsequently absorbed as di- or tripeptides or as amino acids in the human intestine [31]. *In vivo*, peptides can reach immune cells such as lymphocytes and dendritic cells in the gut-associated lymphoid tissue through transcytosis by microfold (M) cells. DCs of the lamina propria could also sample peptides directly from the intestinal lumen by extending their dendrites through the epithelial layer [32]. Both intact κ-CN glycomacropeptide (GMP) and peptides have also been detected in the plasma of adult humans after ingestion of milk or yoghurt [33].

Activation of T cells *in vitro* in the absence of DCs or other APCs is frequently accomplished by anti-CD3/CD28 antibodies coupled to beads. Previous results have shown that a restimulation of activated T cells in the presence of WPCG hydrolysates would affect proliferation differently depending on the activators used. WPCG hydrolysates appeared slightly stimulatory on T-cell proliferation when using anti-CD3/CD28 coated beads while restimulation with anti-CD3 coated beads markedly reduced their proliferation [18]. The present study could not confirm the effect of the WPCG hydrolysates on T cells restimulated with anti-CD3/CD28 coated beads. Rather these hydrolysates displayed a week inhibitory effect on the cells. The cells used in the present study were from a different donor compared to the cells used in the previous study and this could account for some of the differences observed. The effect of the samples on T-cell proliferation restimulated by anti-CD3/CD28 coated beads was in either case only minor, possibly due to the strong signals provided by the costimulatory molecule CD28.

The inhibitory effect of WPCG hydrolysates on anti-CD3-induced proliferation of T cells was, however, similar to that previously reported. Several of the peptides also showed suppressive effects, as did the hydrolysate fractions. The suppressive effect was, however, lower in comparison with the complete WPCG hydrolysate. Thus, we have not yet identified or tested the major antiproliferative component (-s) within the whey protein hydrolysate. Alternatively, the effect of the peptides may be synergistic, caused by the collective action of

peptides in a mixture. None of the hydrolysate samples seemed to negatively affect the amount of viable cells indicating that they are not toxic to the T cells (data not shown). Previously we have also shown that WPCG and its hydrolysates did not induce apoptosis in PBMCs [18]. Nevertheless, the inhibitory effect of the peptides during the anti-CD3-induced proliferation could indicate that they somehow interfere with intracellular activation signals necessary for proliferation.

To further characterise the immunomodulatory effect of the whey peptides, multiplex and ELISA analyses were used to map their effect on the secretion of cytokines from T cells and dendritic cells. In the present study all the samples (hydrolysates and single peptides) increased the T-cell secretion of IL-1β and IL-12 to varying degrees. The effect on the secretion of the other cytokines tested was, however, dependent on the added sample.

To our knowledge no study has looked at the effect of whey protein hydrolysates or single peptides on the secretion of cytokines by DCs. Unlike immature DCs that are mainly able to phagocytose antigens, activated mature DCs are antigen presenting cells able to activate T cells. It was therefore of interest to find out how the secretion of cytokines by mature DCs was affected by the presence of whey peptides, since this could drive the development of the T-cell response. Only the DC secretion of IL-6 and IL-8 was affected by the whey peptide samples. Three β-CN peptides (f41-51, f81-91 and f144-151) increased the secretion of IL-6 while the secretion of IL-8 by DCs was strongly stimulated by all the WPCG HGJ/HDJ fractions and half of the peptides tested. A positive effect on the secretion of IL-8 as well as TNF-α and IL-1β by monocytes incubated with GMP has also been observed [11]. This effect was found to be mediated through stimulation of the mitogen-activated protein kinases (MAPK) and the nuclear factor-kappa B (NF-kB) signal transduction pathways [11].

Recently it was shown that a whey protein isolate and its digest produced by commercial trypsin and chymotrypsin stimulated the proliferation of resting murine splenocytes[8]. It was further shown that peptide fractions (<10 kDa) of the enzymatic digest increased the proliferation of both resting and mitogen stimulated splenocytes. Secretion of IL-2 and IFN-γ by the resting splenocytes was enhanced by both the enzymatic digest and the peptide fractions [8]. Both IL-2 and IFN-γ are important early signals in T-cell activation. We observed that our whey protein hydrolysate and most of the tested peptides increased the secretion of these cytokines, but at the same time inhibited the proliferation of activated T cells. Even though the secretion of IL-2 increased, T-cell proliferation could be inhibited if the expression of high affinity IL-2 receptors are simultanously reduced. GMP has by others been shown to adhere directly to the $CD4^+$ T-cell surfaces and to suppress the expression of the IL-2 receptor [34].

GMP has also demonstrated antiproliferative effects towards activated murine splenocytes *in vitro* [35, 36]. Requena *et al.* [12] also found that GMP inhibited IFN-γ as well as TNF-α production by mitogen activated murine splenocytes. The only whey peptide to reduce the secretion of IFN-γ and IL-2 in the present study was κ-CN f106-124 (N-terminal of GMP). This peptide showed an overall markedly different cytokine secretion profile compared to the shorter peptide κ-CN f109-121. It is therefore likely that the mode of inhibition of T-cell proliferation is different for κ-CN f106-124 compared to the other peptides. We also speculate that it is this part of the GMP that is responsible for the inhibitory effect of GMP on PBMC proliferation observed by others [12, 35, 37]. The synthesised GMP peptides used in the present study are, however, not glycosylated or phosphorylated so the observed effects are caused by their specific amino acid composition.

Most of the cytokines stimulated by the whey peptide samples are pro-inflammatory [38]. Nevertheless, a substantially increased secretion of IL-10 indicates, at least partially, that the whey peptides might have a regulatory function. The incubation of a murine monocytic cell line with GMP has also been shown to induce the expression of an IL-1 receptor antagonist (IL-1ra) [39]. GMP could thereby block the action of the pro-inflammatory IL-1 by binding of the IL-1ra to IL-1 receptors avoiding the activation of splenocyte proliferation and thereby an inflammatory response. GMP has by Requena *et al.* [12] also been shown to up-regulate the expression of the T regulatory cell marker forhead box P3 (Foxp3). In addition, it is important to emphasize that only a selection of the many cytokines that could be affected has been included in the present study. The observed immunomodulatory effect could also possibly be receptor mediated either by binding to surface molecules on the cells or by binding to intracellular molecules. This, however, remains to be studied.

The cytokine profiles of the tested peptides seemed unique for each peptide and the many peptides present in the hydrolysates could display possible synergistic or antagonistic effects. Changing the relative amounts of the different peptides within the hydrolysate, such as by fractionation, could thereby induce different effects.

Conclusion

The present study showed that digestion of whey proteins with human gastric and duodenal juices generated peptides of immunomodulatory nature. None of the tested peptides could, however, completely explain the observed inhibitory effect of the hydrolysate. The different peptides nevertheless displayed both common and unique features on cytokine profiles after incubation with T cells and dendritic cells. Further studies are needed to characterise the mechanisms behind the observed effects; whether the effects are receptor mediated or if peptides will display synergistic or antagonistic effects.

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Tables and figures

Table 1. Overview of the number and size distribution of peptides identified in the caprine whey protein concentrate after digestion with human gastric juice (HGJ) or by HGJ and human duodenal juice (HDJ).

Protein	Gastric digestion (HGJ)			Gastric and duodenal digestion (HGJ/HDJ)		
	Identified peptides	Mw min (Da)	Mw max (Da)	Identified peptides	Mw min (Da)	Mw max (Da)
β-LG	12	950	1843	43	856	3437
β-CN	46	802	2896	25	832	2640
κ-CN	23	899	3647	23	810	2293
α-LA	n.i.			7	1221	1818
SA	9	1104	2443	5	1093	1425
LF	10	1017	2205	6	958	1606

β-LG - β-lactoglobulin; β-CN - β-casein; κ-CN - κ-casein; α-LA - α-lactalbumin; SA - serum albumin; LF - lactoferrin

Table 2. Peptides identified by LC-MS/MS in the caprine whey protein concentrate (WPCG) digested with human gastric (HGJ) and duodenal (HDJ) juices. A cross (x) shows in which fraction the peptides where identified.

Protein	Position	Amino acid sequence	WPCG HGJ/HDJ		
			> 8 kDa	5-8 kDa	< 5 kDa
β-LG	1-8	IIVTQTMK	x		
β-LG	9-18	GLDIQKVAGT			x
β-LG	21-32	SLAMAASDISLL	x		
β-LG	33-40	DAQSAPLR	x		
β-LG	43-55	VEELKPTPEGNLE			x
β-LG	71-82	IIAEKTKIPAVF	x		
β-LG	92-100	VLVLDTDYK			x
β-LG	125-134	TPEVDKEALE		x	
β-LG	139-147	ALKALPMHI	x		
β-LG	149-159	LAFNPTQLEGQ		x	x
β-CN	1-9	REQEELNVV			x
β-CN	41-51	TEDELQDKIHP			x
β-CN	61-72	PFTGPIPNSLPQ			x
β-CN	81-91	PVVVPPFLQPE		x	x
β-CN	99-105	KETMVPK			x
β-CN	144-151	MHQPPQPL			x
β-CN	191-205	YQEPVLGPVRGPFPI			x
κ-CN	106-124	MAIPPKKDQDKTEIPAINT			x
κ-CN	109-121	PPKKDQDKTEVPA		x	x
κ-CN	126-133	ASAEPTVH			x
κ-CN	130-139	PTVHSTPTTE		x	
κ-CN	141-153	IVNTVDNPEASSE		x	x

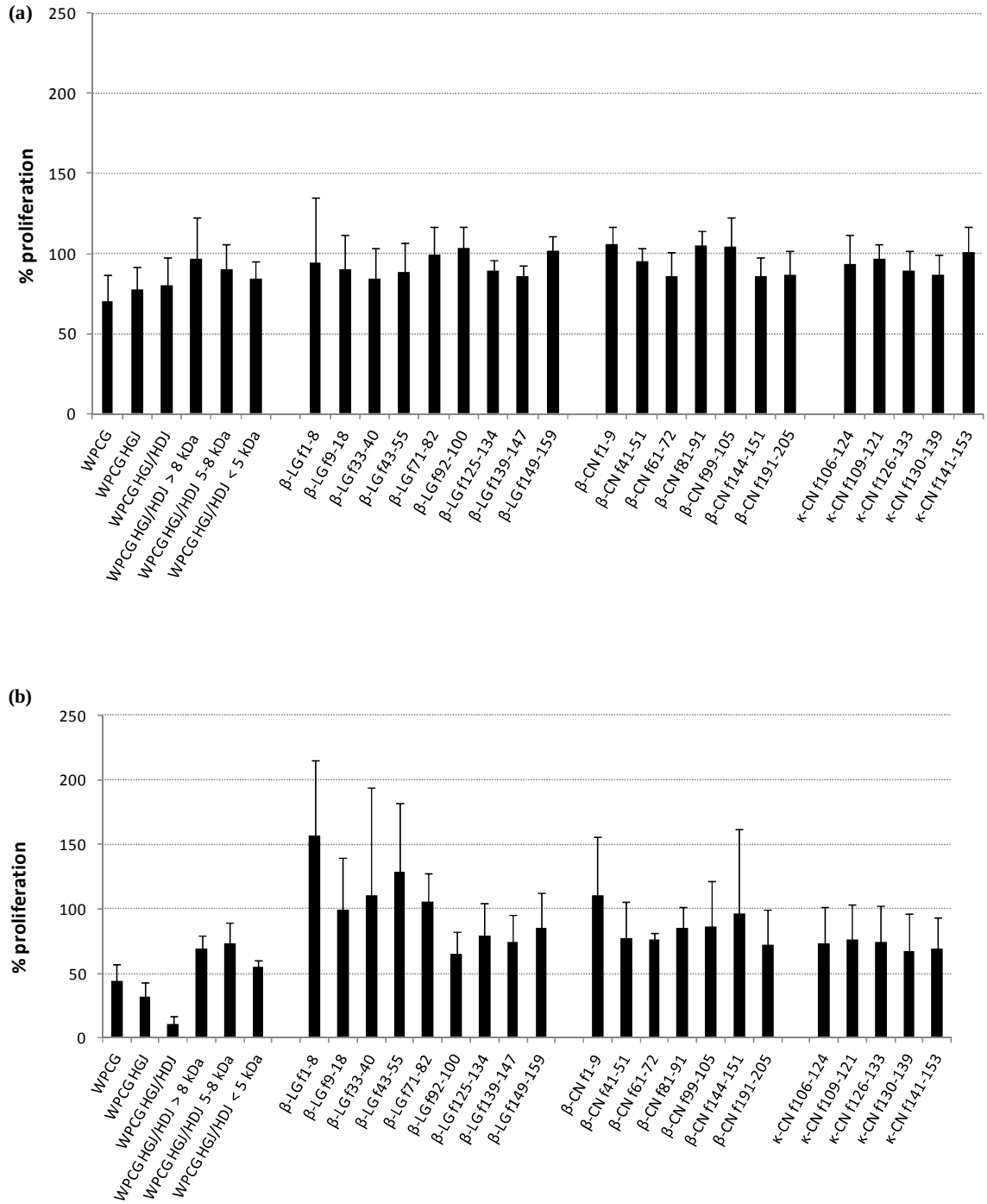

Figure 1. Effect of whey protein samples on the proliferation of T cells restimulated using (a) anti-CD3/ CD28 coated beads or (b) anti-CD3 coated beads. While anti-CD3/CD28 coated bead provides powerful activation signal that may mask the effect of the peptides, apparent effects of the whey protein samples is seen when restimulating with anti-CD3 coated bead. Average numbers with standard deviations for 3-5 assays is shown relative to the control cells with no added whey protein/ peptides. Abbreviations: Caprine whey protein concentrate (WPCG), human gastric juice (HGJ), human duodenal juice (HDJ), β-lactoglobulin (β-LG), β-casein (β-CN) and κ-casein (κ-CN).

Table 3. Overview of the average cytokine response induced by the addition of whey peptides and hydrolysate fractions to T cells activated by anti-CD3/CD28 coated beads.

		T-cell cytokine secretion									
Peptide		IL-1β	IL-2	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12	MCP-1	IFN-γ
WPCG HGJ/HDJ > 8 kDa		79	26	7	9	26	31	22	65	6	25
WPCG HGJ/HDJ 5-8 kDa		56	12	-6	-2	26	44	3	131	-5	5
WPCG HGJ/HDJ < 5 kDa		81	44	4	29	36	57	54	156	56	55
β-LG f1-8	IIVTQTMK	110	28	5	15	49	24	25	101	38	42
β-LG f9-18	GLDIQKVAGT	41	23	3	17	24	7	37	22	22	22
β-LG f33-40	DAQSAPLR	107	19	-15	11	51	7	-3	45	14	0
β-LG f43-55	VEELKPTPEGNLE	96	21	9	2	41	14	44	86	20	37
β-LG f71-82	IIAEKTKIPAVF	80	28	25	25	43	31	84	70	36	66
β-LG f92-100	VLVLDTDYK	52	39	13	14	20	15	37	50	18	41
β-LG f125-134	TPEVDKEALE	30	2	-1	-7	15	-5	33	53	-11	28
β-LG f139-147	ALKALPMHI	19	22	7	-8	0	6	22	34	-27	11
β-LG f149-159	LAFNPTQLEGQ	80	11	7	3	33	15	52	84	27	38
		41	16	-3	-16	11	17	51	60	-1	36
β-CN f1-9	REQEELNVV	38	9	-14	-20	13	26	65	11	15	28
β-CN f41-51	TEDELQDKIHP	26	0	0	-11	-4	5	8	68	-1	7
β-CN f61-72	PFTGPIPNSLPQ	47	22	1	3	15	14	52	61	5	31
β-CN f81-91	PVVVPPFLQPE	55	18	-2	-3	20	33	38	71	17	21
β-CN f99-105	KETMVPK	66	10	0	-5	19	11	46	36	24	23
β-CN f144-151	MHQPPQPL	81	27	19	34	26	18	61	35	25	45
β-CN f191-205	YQEPVLGPVRGPFPI	66	12	0	7	25	30	78	110	30	64
κ-CN f106-124	MAIPPKKDQDKTEIPAINT	23	-10	-18	-11	-3	39	5	53	3	-6
κ-CN f109-121	PPKKDQDKTEVPA	58	48	18	22	23	29	44	133	23	49
κ-CN f126-133	ASAEPTVH	30	20	-1	1	8	71	19	54	2	11
κ-CN f130-139	PTVHSTPTTE	14	16	8	4	7	19	10	16	4	27
κ-CN f141-153	IVNTVDNPEASSE	66	30	3	-4	13	21	38	97	17	45

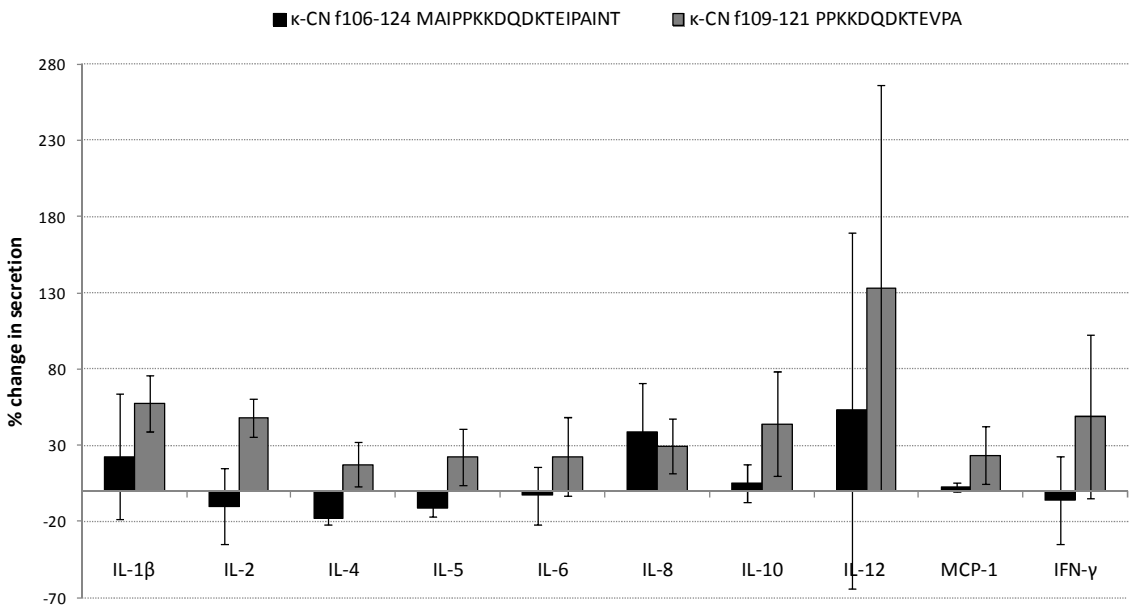

Figure 2: Comparison of the cytokine profiles of human T cells activated by anti-CD3/CD28 coated beads and incubated with κ-casein (κ-CN) f106-124 or f109-121.

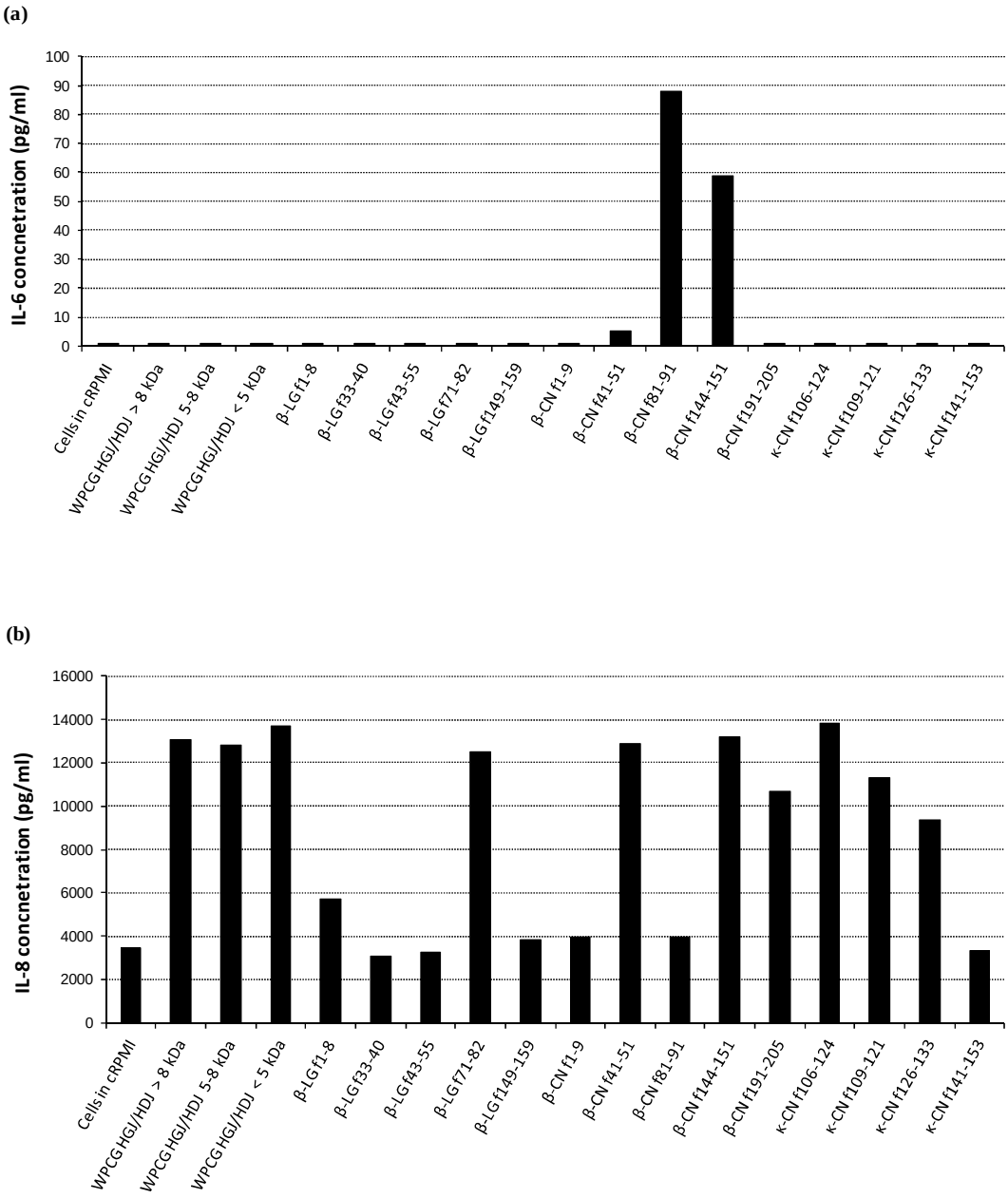

Figure 3: Effect of whey protein hydrolysates and single peptides on the production of (a) IL-6 and (b) IL-8 in LPS-activated human dendritic cells.