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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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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 activity (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].

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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.

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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 $P < 0·05$. 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.

[IIVTQTMK]GLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
[IIVTQTMKGLDIQ]KVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
[IIVTQTMKGLDIQK]VAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
[IIVTQTMKGLDIQKVAGTWY]SLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
[IIVTQTMKGLDIQKVAGTWYSLAMAASD]ISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
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IIVTQTMK[GLDIQKVAGTWYSLA]MAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
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IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVY[VEELKPTPEGNLEILLQ]KWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKK[IIAEKTKIPAVF]KIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVF[KIDALNENK]VLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVF[KIDALNENKVL]VLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDAL[NENKVLVLDTD]YKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENK[VLVLDTDYK]KYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCL[VRTPEVDKEALEKFD]KALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALE]KFDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALEK]FDKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALEKF]DKALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALEKFD]KALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALEKFDK]ALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVR[TPEVDKEALEKFDKALKALPMHI]RLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDK[EALEKFDK]ALKALPMHIRLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDK[ALKALPMHI]RLAFNPTQLEGQCHV
IIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEGNLEILLQKWENGECAQKKIIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLACQCLVRTPEVDKEALEKFDKALKALPMHIR[LAFNPTQLEGQ]CHV

Fig. 1. Full-length amino acid sequence of β-lactoglobulin and identified peptides (forty-three framed) generated by digestion with human gastrointestinal enzymes from human gastric juice (30 min) and human duodenal juice (30 min) at 37°C.

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REQEELNVVGETVESLSSSEESITHINKKIEKFQSEEQQQTEDELQDKIHPFAQAQSLVYPFTGPIPNSLPQNILPLTQTPVVVPPFLQPEIMGVPKVKETMVPKHKEMPFPKYPVEPFTESQSLTLTDVEKLHLPLPLVQSWMHQPPQPLSPTVMFPPQSVLSLSQPKVLPVPQKAVPQRDMPIQAFLLYQEPVLGPVRGPFPILV

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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MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTE**V**PAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTE**E**PAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTE**M**PAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTE**P**PAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
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MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV
MAIPPKKDQDKTEIPAINTIASAEPTVHSTPTTEAIVNTVDNPEASSESIASASETNTAQVTSTEV

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

APRKNVRWCAISLPEWSKCYQWQRRMRKLGA
PSITCVRRTSALECIRAIAGKNADAVTLDSGMVFEAGLDPYKLRPVA**AEI**
YGTEKSPQTHYYAVAVVKKGSNFQLDQLQGQKSCHAGLGRSA**GWNIPVGI**
LRPFLSWT**ESAEPLQGAVARF**FSASCVPCVDGKAYPNLCQLCKGVGENKC
ACSSQEPYFGYSGAFKCLQDGAGDVAF**VKETTVFENLPEKADRDQY**ELLC
LNNTRAPVDAFKECHLAQVPSHAVVA**RSVDGKENL**IWEL**LRKAQEKFGKN**
KSQRFQLFGSPEGRRDLLFKDSALGF**VRIPSKVDSA**LYLGSRYLTALKNL
RETAEEVKARCTRVVWCAVGPEEQSKCQQWSEQSGQNVTCATASTTDDCI
ALVLKGEADALSLDGGYIYTAGKCGLVPVMAENRKSSKYSSLDCVLRPTE
GYLAVAVVKKANEGLTWNSLKGKKSCHTAVDRTAGWNIPMGLIANQTGSC
AFDEFFSQSCAPGADPKSSLCALCAGDDQGLDKCVPNSKEKYYGYTGAFR
CLAEDVGDVAFVKNDTVWENTNGESSADWAKNLNREDFRLLCLDGTTKPV
TEAQSCYLAVAPNHAVVSRSDRAAHVEQVLLHQQALFGKNGKNCPDQFCL
FKSETKNLLFNDNTECLAKLGGRPTYEKYLGTEYVTAIANLKKCSTS**PLL**
EACAFLTR

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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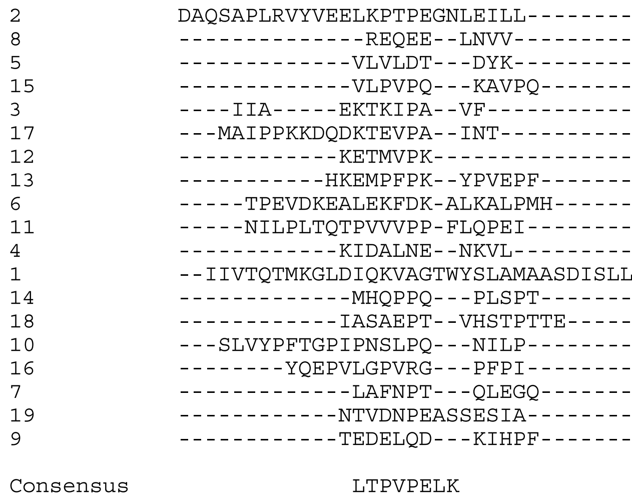

Fig. 5. Clustal multiple sequence alignment of nineteen peptides. Peptides no. 1–7 are derived from β-lactoglobulin, no. 8–16 from β-casein and no. 17–19 from κ-casein glycomacropeptide. The consensus sequense, LTPVPELK, is shown with leucine (L), proline (P) and valine (L).

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.

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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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