Release of the anti-hypertensive tripeptides valine-proline-proline and isoleucine-proline-proline from bovine milk caseins during *in vitro* gastro-intestinal digestion

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Abstract

The aim of this study was to identify and quantify the release of antihypertensive tripeptides valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) during *in vitro* oro-gastro-intestinal (OGI) digestion of bovine skimmed milk. The experimental approach combined the recently developed harmonized static *in vitro* digestion (IVD) model and targeted mass spectrometry in order to monitor peptide generation. We firstly demonstrated that VPP and IPP are released from bovine milk proteins during *in vitro* OGI digestion at final concentrations of 354.3 ± 29.8 and 973.8 ± 155.7 µg/L, respectively. *In silico* analysis of cleavage sites and mass spectrometry revealed that tetrapeptides VPPF, IPPL and IPPK are precursors of VPP and IPP. The release of other ACE-inhibitory peptides, such as FVAP, VAP, AW and VY, was demonstrated and their fate and the time course were investigated. This research underlines the suitability of IVD system to study the release of short bioactive peptides during OGI transit.

Keywords: milk; IPP; VPP; mass spectrometry; *in vitro* human digestion.

Introduction

Bovine milk proteins contains encrypted in their sequences several bioactive peptides with demonstrated biological functionalities such as anti-hypertensive, antioxidant, immunomodulatory and anti-microbial activities.[1]

Among the milk-derived bioactive peptides, the anti-hypertensive lacto-tripeptides valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) have attracted particular attention in the last years.[2,3] They have shown potentiality as anti-hypertensive agents due to their inhibitory effects on angiotensin-converting enzyme (ACE) showing IC_{50} values of 9 and 5 μmol/L, respectively.[4] Furthermore, although several *in vivo* studies confirmed the antihypertensive effect of the two lactotripeptides in spontaneously hypertensive rats (SHR),[5,6] the results from human clinical trials are still controversial. In 2012 the European Food Safety Authority (EFSA) published an opinion of the Panel on Dietetic Products, Nutrition and Allergies on the health claims related to IPP and VPP, with special regards to the maintenance of normal blood pressure.[7] The EFSA identified 25 human intervention studies, 15 of which did not observe any effect of IPP and VPP on systolic or diastolic blood pressure. However, three recent meta-analyses of human clinical trials demonstrated a small but significant lowering effect on systolic and diastolic blood pressure, especially in pre-hypertensive or mildly hypertensive patients.[8-10] The exact mechanism of anti-hypertensive activity of VPP and IPP is still not known and may involve, other than the ACE-inhibition, the production of vasodilators such as nitric oxide [11,12] or an effect on sympathetic nervous activity.[13] A recent study also demonstrated that VPP and IPP exhibit insulin-like activities on cultured adipocytes and are able to suppress cytokine-mediated inflammatory responses in the same cell line, suggesting the potential use of these lactotripeptides in the management of metabolic syndrome and its complications.[14] Lactotripeptides VPP and IPP are easily released from bovine caseins by starter lactic acid bacterium *Lactobacillus helveticus* owing to its repertoire of cell wall bound proteinases and cytoplasmic peptidases able to hydrolyze caseins to VPP and IPP.[2] Recently, strains of the non-starter species *Lactobacillus casei* and *Lactobacillus rhamnosus* were also found to be able to

release VPP and IPP during milk fermentation.[15] Thanks to the proteolytic activities of these lactobacilli, VPP and IPP were frequently detected in several fermented food, such as hard and soft cheeses.[16] Therefore, fermentation processes driven by lactobacilli appear to be the primary way for releasing VPP and IPP from bovine caseins. The milk-derived lacto-tripeptides VPP and IPP are the active ingredients of the sour milk Calpis (Calpis Co., Tokyo, Japan) and Evolus (Valio, Helsinki, Finland), produced by fermentation with *Lactobacillus helveticus*.[2]

These tripeptides have been shown to be highly resistant to the proteases secreted in the oro-gastro-intestinal (OGI) tract and to the brush border peptidases.[16] Moreover, IPP was found to be bioavailable after oral consumption of a yogurt beverage enriched in antihypertensive peptides.[17]

Some evidences suggest the possible release of IPP from intact protein or large peptide sequences in the intestinal tract. Foltz et al.[17] found plasma IPP concentrations significantly higher than the baseline concentrations after consumption of the placebo beverage instead the lacto-tripeptide enriched beverage. The placebo beverage did not contain free IPP but only intact bovine milk proteins, suggesting that IPP was generated in the intestinal tract of human subjects. Furthermore, we recently showed that IPP was released from camel κ-casein during *in vitro* digestion.[18]

However, no demonstrations have been given so far about the release of VPP and IPP from bovine caseins by mammalian OGI proteolytic enzymes.

Ohsawa et al.[19] studied the *in vitro* release of bioactive peptides from bovine caseins using a mammalian model of the gastro-intestinal tract. Despite they have found in the digested milk several precursors of VPP and IPP, the presence of the lacto-tripeptides was not confirmed. The study however suffered of some limitations. The gastro-intestinal model was far from the physiological conditions and the software used for peptide identification were not able to detect peptides of length less than four or five amino acids.[20,21]

Recently, an *in vivo* study carried out by Boutrou and colleagues[22] confirmed the presence of numerous precursors of VPP and IPP in the jejunum of healthy subjects following caseins intake.

Unfortunately, the lacto-tripeptides were not detectable under the condition used in the mass spectrometry analysis by Boutrou et al.[22]

Simulated gastro-intestinal digestion models are widely employed in many fields of food and nutritional sciences and are useful tools to study, in a simplified manner, the digestion process in the upper gastro-intestinal tract applying physiological-based conditions, i.e., chemical and enzymatic compositions of digestive fluids, pH and residence periods typical for each compartment. Recently, a basic static *in vitro* digestive (IVD) model simulating human digestion has been developed within the COST action INFOGEST[20] with the aim to harmonize inter-laboratory results and to set experimental conditions that are most close as possible to the physiological situation. The harmonized IVD system has been successfully utilized to study the release of antihypertensive peptides from camel milk[18] and gluten-derived sequences from pasta[23], as well as the bioaccessibility of β-carotene[24] and phenolic compounds[25].

The aim of this study was to investigate the release and fate of the anti-hypertensive tripeptides VPP and IPP and some other ACE-inhibitory peptides during simulated OGI digestion of bovine skimmed milk. Differently from the previous efforts to detect VPP and IPP in milk digestates[19,22] in the present work, we combined an *in vitro* OGI digestion system, which exploited the same conditions of the harmonized IVD system developed within the COST action INFOGEST,[20] with targeted mass spectrometry analysis.

Materials and methods

Materials

Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine α-amylase, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP), mucin II and III, bovine serum albumin, urea and trinitrobenzensulfonic acid (TNBS) were supplied by Sigma (Milan, Italy). The tripeptides VPP and IPP (95% purity) were synthesized by DBA (Milan, Italy). Amicon Ultra-0.5 mL regenerated cellulose 3 kDa were supplied by Millipore (Milan, Italy). Ultra-high-temperature-treated (UHT) skimmed bovine milk was purchased from a local market (Reggio Emilia). Mass spectrometry solvents and all the other reagents were purchased from Carlo Erba (Milan, Italy). The absorbance values were determined through a Jasco V-550 UV/Vis spectrophotometer (Orlando, FL, U.S.A.).

In vitro gastro-intestinal digestion of skimmed milk using harmonized protocol

For the *in vitro* digestion, the protocol previously developed within the COST Action INFOGEST and further validated for milk, was used.[20,26,27] Briefly, simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were prepared according to Kopf-Bolanz et al.[26] Intestinal fluid was prepared by mixing pancreatic (PF) and bile (BF) fluids to final ratio 2:1 (v/v). Skimmed bovine milk (18 mL) was mixed with 24 mL of SSF containing 150 U/mL of porcine α-amylase and incubated for 5 min (oral phase). Gastric phase was mimed by adding 48 mL of SGF to the digestion mixture, adjusting the pH to 3.0 with 6.0 N HCl, and supplementing porcine pepsin to achieve the final concentration of 1115 U/mL. The resulting gastric chyme was further incubated for 120 min. For intestinal phase, 72 mL of SIF (48 mL of PF and 24 mL of BF) was added to the gastric chyme, the pH was adjusted to 7.0 with NaOH 1N and pancreatin was supplemented at the final concentration of 0.4 mg/mL (corresponding to a final trypsin activity of 120 U/mL as suggested by Minekus et al.[20]). The digestion mixture was further incubated for 180 min (pancreatic phase). All incubations were performed at 37 °C on a rotating wheel (10 rpm). The digested

samples were sampled at 12 time points (after 0 and 5 min of salivary digestion, after 30, 60, 90 and 120 min of gastric digestion and after 20, 30, 60, 90, 120, 180 min of intestinal digestion, respectively), cooled on ice and immediately frozen at –80 °C for further analyses. The digestions were performed in triplicate.

A control sample, which consisted of the gastro-intestinal juices, enzymes and water in place of milk, was included in the experimental trials to evaluate the possible impact of the digestive enzymes on the subsequent analyses.

Determination of protein hydrolysis during the digestion

The determination of protein hydrolysis in the digested samples was carried out by measuring the peptide concentration by the TNBS method using leucine as standard.[28]

The hydrolysis degree (DH) was expressed in percentage and calculated as reported in equation (1):

$$\mathbf{DH(\%) = (h/h_{tot}) \cdot 100} \quad (1)$$

where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of α-amino groups formed at the different stages of the simulated digestion, and $\mathbf{h_{tot}}$ is the hydrolysis equivalent at complete hydrolysis to amino acids (calculated by summing the contents of the individual amino acids in 1 g of protein and considering caseins as the only proteins in milk). According to Adler-Nissen,[28] the $\mathbf{h_{tot}}$ value was fixed at 8, which is the value calculated for caseins. DH data were subtracted with the data obtained in the control digestion.

Nanoflow liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization (LC-ESI-QTOF-MS/MS) analysis

Samples (0.5 mL) collected during the *in vitro* digestion were subjected to ultrafiltration with Amicon Ultra-0.5 mL nominal cut-off 3 kDa, at 12000*g* for 120 min at 4 °C.

Nano LC/MS and tandem MS experiments were performed on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS via a

Chip Cube Interface (Agilent Technologies, Santa Clara, CA, U.S.A.). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4mm 40 nL enrichment column and a 43 mm × 75 μm analytical column, both packed with a Zorbax 300SB 5 μm C18 phase (Agilent Technologies). The mobile phase composition and the gradient were the same as reported by Tagliazucchi et al.[29] The mass spectrometer was tuned, calibrated and set with the same parameters as reported by Dei Più et al.[30]

Monoisotopic precursor selection was applied to identify the lactotripeptides, some possible precursors and additional ACE-inhibitory peptides. The assignment process was complemented and validated by the manual inspection of MS/MS spectra. The sequences of the peptides studied are listed in **Table 1** together with the selected precursor and the product ions.

VPP and IPP was quantified using the method reported in Solieri et al.[15] and their amount expressed as μg/L of hydrolysates. All of the other selected peptides were quantified by integrating the area under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide.

Statistical analysis

The samples obtained from each digestion were analyzed in singular by TNBS assay and by mass spectrometry. All data are presented as mean ± SD. Univariate analysis of variance (ANOVA) with Tukey *post-hoc* test was applied using Graph Pad Prism 6.0 (GraphPad Software, San Diego, CA). The differences were considered significant with $P < 0.05$.

Results and Discussion

Assessment of protein hydrolysis during simulated digestion

The hydrolysis of bovine milk proteins during the *in vitro* digestion was evaluated with the TNBS assay. **Figure 1** reports the resulting DH values, relative to control condition, at various steps of the simulated OGI transit. As expected, simulated salivary digestion did not enhance the DH. No significant differences were found between the DH values before and after salivary digestion. After 30 min of gastric digestion, the DH slightly increased from 4.3 ± 0.3% to 10.2 ± 2.4% (**Figure 1**). Major but not significant increase in DH was observed during the subsequent 90 min of gastric digestion, reaching the value of 12.2 ± 2.2% at the end of gastric phase. The transition from gastric to pancreatic treatment determined a significant enhance of DH values (26.4 ± 2.9% after 20 min of pancreatic digestion; $P<0.0001$) (**Figure 1**). Subsequently, the DH showed a tendency to gradually increase. Degradation of protein occurs mainly in the intestinal fluid due to the digestive action of pancreatin that is a mix of different proteases. After 120 min of intestinal incubation, DH reached the plateau (57.4 ± 12.7%) (**Figure 1**).

The DH values measured after 120 min of simulated gastro-pancreatic digestion are in agreement with those previously determined on similar milk-derived protein substrates and with the same harmonized digestion model.[26,31] In particular, comparison between bovine and camel milks showed that bovine milk proteins are more resistant to OGI digestion than camel milk proteins. Complete digestion of camel milk proteins with the same harmonized protocol resulted in a DH value of 69.6 ± 2.1%[18] owing to the higher susceptibility of camel milk proteins to peptic digestion compared to bovine milk proteins.[18] The DH value measured after peptic hydrolysis of bovine and camel milk proteins, digested with the same harmonized digestion model, was 12.2% and 20.5%, respectively.[18]

Release of valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) from bovine milk during in vitro digestion

Even if different studies have suggested that bioactive peptides released from milk protein during digestion could exert *in vitro* ACE-inhibitory activity,[22] no evidence has been given until now about the possible release of the anti-hypertensive peptides VPP and IPP from bovine milk protein during *in vitro* OGI digestion. The only study aimed at the identification of VPP and IPP after gastro-intestinal digestion failed to detect the lacto-tripeptides in the intestinal digesta.[19]

Bovine caseins have encrypted in their sequence both the lactotripeptides VPP and IPP. This last tripeptide was located both in β- and κ-caseins (fragments 74-76 and 108-110, respectively), whereas VPP was found only in β-casein (fragment 84-86). The producibility of these tripeptides by sequential digestion with pepsin and pancreatic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and B) was evaluated by analyzing the hydrolyzates collected at different times of simulated OGI digestion with nanoflow-LC-ESI-QTOF-MS/MS. VPP and IPP were not detected at the end of the gastric phase, whereas the addition of the pancreatic enzymes determined the release of both the antihypertensive lactotripeptides. As reported in **Figure 2**, their amounts tended to increase during the intestinal phase. In particular, IPP amount reached 414.6 ± 11.3 μg/L of hydrolysates after the first 20 minutes of intestinal phase, and remained stable for the successive 70 minutes of digestion. After this time, the concentration of IPP sharply increased until 180 minutes of digestion without reaching a plateau. In contrast, the VPP concentration exhibited a linear trend of increase during the first 60 minutes of digestion, did not change significantly during the pancreatic digestion, and raised during the late intestinal digestion. At the end of simulated digestion, IPP and VPP amounts were 973.8 ± 155.7 and 354.3 ± 29.8 μg/L of hydrolysates, respectively (**Figure 2**). Nineteen randomized clinical intervention trials showed that daily doses (2–10 mg) of milk casein-derived lactotripeptides reduce the systolic (4.0 mmHg) and diastolic (1.9 mmHg) blood pressure in hypertensive patients.[10] Based on our data, a daily consumption of 300 mL of skimmed milk would be sufficient to obtain an intake of VPP and IPP of about 4 mg.

Simulated OGI digestion demonstrated that IPP is released from milk caseins at higher amounts than VPP. The κ- and β-caseins in bovine milk represents the 9.3% and 33.7% of the total milk proteins, respectively.[32] As the protein concentration in the digestive system was 3.9 g/L (35 g/L in milk, 9-fold diluted with digestive fluids), the κ- and β-casein concentrations were estimated about 363 and 1314 mg/L, respectively (corresponding to 19.2 and 54.8 μmol/L, respectively). Considering that 2.99 μmol/L of IPP was found at the end of the digestion and that IPP is present in both κ- and β-caseins, the recovery yield of IPP was approximately 4.0%. Similarly, considering that VPP is present only in β-casein, the recovery yield was approximately 2.1%, given the final VPP concentration of 1.14 μmol/L. Furthermore, the amount of IPP released from bovine milk was about 4 times more than that released from camel milk after 120 min of pancreatic digestion.[18] However, taken into consideration that IPP is present only in position 100-102 of camel milk κ-casein, the recovery yield of IPP from camel milk is about 4-fold higher than that obtained from bovine milk.[18]

Reconstruction of possible release pathway of VPP and IPP during intestinal digestion

As detailed in **Figure 3,** *in silico* analysis of theoretical cleavage sites in the VPP or IPP–containing sequences of bovine milk caseins suggested that several digestive proteolytic enzymes might be involved in releasing these tripeptides. In particular, pepsin was found to cleave preferentially bonds with aromatic residues, such as F, Y and W, or L in position P1 and P1'.[33] Therefore, possible cleavage sites in the long sequence 70-92 of β-casein are L_{70}—P_{71}, L_{77}—T_{78}, F_{87}—L_{88} and L_{88}—Q_{89}. Schmelzer et al.[34] found additional cleavage sites for pepsin in β-casein, such as I_{74}—P_{75}, T_{80}—P_{81}, V_{83}—V_{84} and V_{84}—P_{85}. The peptide bonds N_{72}—I_{73}, L_{77}—T_{78} and F_{87}—L_{88} can be also hydrolyzed by chymotrypsin which preferentially cleaves at W, Y and F in position P1 and to a lesser extent at L, M and N in position P1.[33] Elastase shows specificity towards A, V, S and L in position P1,[35] and could be involved in the hydrolysis of peptidic bonds L_{77}—T_{78}, V_{83}—V_{84} and

V_{84}—P_{85}. None of these endoproteases should be able to hydrolyze the bonds P_{76}—L_{77} and P_{86}—F_{87}.

Based on these considerations, the tripeptides IPP and VPP can be released from β-casein in the form of the tetrapeptides IPPL and VPPF (**Figure 3**). The C-terminal residues L_{77} and F_{87} can be subsequently removed by pancreatic carboxypeptidase A (C-terminal exopeptidase) releasing the tripeptides IPP and VPP. Bovine κ-casein contains IPP in position 108-110. The peptide bond A_{107}—I_{108} can be easily hydrolyzed by pepsin or elastase, whereas the peptide bond K_{110}—K_{111} is a cleavage site for trypsin. The action of these enzymes should result in the release of the tetrapeptide IPPK (**Figure 3**). The residue K_{110} can be removed by the action of the pancreatic carboxypeptidase B (C-terminal exopeptidase) which cleaves specifically C-terminal K and R residues.

To confirm the pathway of IPP and VPP release reconstructed by *in silico* analysis, three putative precursors (IPPL, IPPK and VPPF) and three possible alternative by-products of hydrolysis (PPL, PPF and PPK) were tentatively identified and quantified by nanoflow-LC-ESI-QTOF-MS/MS during the intestinal digestion (**Figure 4**). Among the three selected precursors, only VPPF was found at the end of the gastric digestion, confirming that the bonds V_{83}—V_{84} and F_{87}—L_{88} are cleavage sites for pepsin. The amount of VPPF gradually increased in the hydrolysates during the pancreatic digestion thanks to the action of elastase and chymotrypsin. VPPF concentration reached a peak after 120 minutes of pancreatic digestion and then decreased in the remaining digestion time. The decrease in VPPF amount after 120 of minutes coincides with the observed increase in VPP concentration (**Figure 2**). Both IPPL and IPPK were released only during intestinal phase of digestion following different trends (**Figure 4**). The amount of IPPL gradually increased during the entire time of digestion without reaching a plateau, whereas IPPK reached the maximum amount after 90 minutes of digestion. Overall, these data confirmed that the tetrapeptides VPPF, IPPL and IPPK are precursors of IPP and VPP, probably due to the cleavage by pancreatic carboxypeptidases. Regarding the alternative by-products of the hydrolysis, PPL, PPF and PPK can be released instead of IPP and VPP and represent un-desired peptides, which reduce the recovery yields of the anti-

hypertensive tripeptides. As shown in **Figure 4**, only PPF was found in the hydrolysates. Trace amount of PPF were found at the end of the gastric digestion, suggesting that pepsin is able to cleave the bond V_{84}—P_{85} with low efficacy. Subsequently, the amount of PPF greatly increased during pancreatic digestion because of the action of elastase. No evidence for the formation of PPL and PPK was found either during gastric or pancreatic digestions, suggesting that the bond I_{74}—P_{75} is resistant to gastro-pancreatic proteases. This result could account for the higher recovery yield observed for IPP compared to VPP.

Time course and fate of additional ACE-inhibitory peptides

The fate of additional four selected peptides with known ACE-inhibitory activity was monitored during the pancreatic phase of digestion. FFVAP (f23-27) and VAP (f25-27) are α_{S1}-casein derived peptides which display high ACE-inhibitory activity and low IC_{50} values (IC_{50} values of 6 and 2 µmol/L, respectively) [36,37]. The pentapeptide FFVAP was firstly isolated from casein sequentially hydrolyzed with trypsin and prolyl-endopeptidase,[36] whereas the tripeptide VAP was chemically synthesized to reproduce the C-terminal portion of a fragment-peptide derived from an enzymatic hydrolysate of casein[37]. However, until now, these ACE-inhibitory peptides were never found in *in vitro* digestive hydrolysates of milk proteins. **Figure 5** shows that combination of the harmonized IVD model with targeted mass spectrometry enables the detection of both peptides already at the end of the gastric digestion. The amount of FFVAP increased until 120 minutes of pancreatic digestion, after that it was further degraded to the shorter tripeptide VAP.

The ACE-inhibitory dipeptides AW and VY have been proved to exhibit low IC_{50} values (5 µmol/L for both the peptides) and to be absorbed in human plasma.[38] AW was found only in α_{S1}-casein (f163-164), whereas VY was found in β-casein (f59-60), α_{S2}-casein (f198-199) and in β-lactoglobulin (f41-42). As reported in **Figure 5**, neither AW nor VY were detected at the end of gastric digestion, but they were released from milk proteins by intestinal proteases. We recovered

286 the maximum amount of AW after 20 minutes of digestion, after that, the amount of AW dropped
287 and did not significantly change during the remaining time of digestion (**Figure 5**). The trend of VY
288 production was different. **Figure 5** shows a biphasic release consisting of a first maximum value
289 after 20 minutes of intestinal digestion, followed by a decrease during the subsequent 60 minutes of
290 digestion. During the successive 30 minutes of digestion, there was a threefold increase of VY
291 amount, followed by a further decrease in the last phase of digestion. The biphasic release of VY is
292 probably due to the presence of this dipeptide in the sequences of different milk proteins, which can
293 release VY thanks to the action of different pancreatic enzymes and at different digestion times.
294 Previous studies based on simplified and in-house digestive models failed to detect short bioactive
295 peptides in the digested milk. Here, we demonstrated that the harmonized gastro-intestinal IVD
296 model in combination with targeted mass spectrometry enables the identification of short ACE-
297 inhibitory and anti-hypertensive peptides released during digestion of milk proteins. Therefore, the
298 harmonized digestive system is confirmed to be an effective model to study the fate, kinetics of the
299 release and concentrations of short bioactive peptides during milk digestion in a reproducible
300 manner.
301 As a major result, our study provides the first evidence that VPP and IPP are released during the
302 OGI digestion of bovine milk caseins. The recovery yield of the two lactotripeptides was low for
303 both VPP and IPP at the first stages of OGI transit, but, as the digestion proceeds, they are released
304 to a greater extent from precursors present in the jejunum. Furthermore, to the best of our
305 knowledge we firstly demonstrate that other short ACE-inhibitory peptides, such as AW, VY, VAP
306 and FFVAP are present in the gastro-intestinal digested milk. Even if further investigation and *in*
307 *vivo* trials are required to corroborate our results, the present work opens the way to study the fate
308 of VPP, IPP and other bioactive peptides under *in vitro* digestive conditions most close as possible
309 to human physiology.

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Figure captions

Figure 1. Changes in hydrolysis degree (DH%) of bovine skimmed milk proteins during *in vitro* gastric and pancreatic digestion. Values are means of three independent digestion ± standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 2. Release of the antihypertensive lactotripeptides valine-proline-proline (VPP) and isoleucine-proline-proline (IPP) during intestinal digestion. Time zero represents the sample collected at the end of the gastric digestion. Values are means of three independent digestion ± standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 3. Theoretical cleavage sites of pepsin (▲), chymotrypsin (●), elastase (♦), and trypsin (■) in the VPP and IPP-containing sequences of bovine β- and κ-caseins.

Figure 4. Fate and time course of the VPP and IPP precursors (VPPF, IPPL and IPPK) and of the un-desired product of hydrolysis PPF during intestinal digestion. Amounts are expressed as area under the peak (AUP). The end of the gastric digestion is considered as the starting point of sampling. Values are means of three independent digestion ± standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$).

Figure 5. Fate and time course of short ACE-inhibitory peptides during intestinal digestion. Amounts are expressed as area under the peak (AUP). The end of the gastric digestion is considered as the starting point of sampling. Values represent means ± SD of triplicate digestions. Different letters indicate significantly different values ($P < 0.05$).

Table 1. Sequences, Precursor Ions Selected for Fragmentation and Monitored Product Ions of the Peptides Identified and Quantified Using Mass Spectrometry.

Peptides	Fragments	Selected precursor ions[a]	Monitored product ions[a]
	β-casein		
VY	f(59-60)	281.15	182.08
IPP	f(74-76)	326.21	213.12; 211.14
IPPL	f(74-77)	439.29	326.21; 308.20; 229.15
PPL	f(75-77)	326.21	229.15; 195.11
VPP	f(84-86)	312.19	213.12; 197.13
VPPF	f(84-87)	459.26	360.19; 294.18; 263.14
PPF	f(85-87)	360.19	263.14; 195.11
	α_{S1}-casein		
FFVAP	f(23-27)	580.31	433.24; 286.18; 187.11
VAP	f(25-27)	286.18	187.11; 171.11
AW	f(163-164)	276.13	205.10
	α_{S2}-casein		
VY	f(198-199)	281.15	182.08
	κ-casein		
IPP	f(108-110)	326.21	213.12; 211.14
IPPK	f(108-111)	454.30	341.22; 308.20; 244.17
PPK	f(109-111)	341.22	244.17; 195.11
	β-lactoglobulin		
VY	f(41-42)	281.15	182.08

a: mono-charged ions

Figure 1

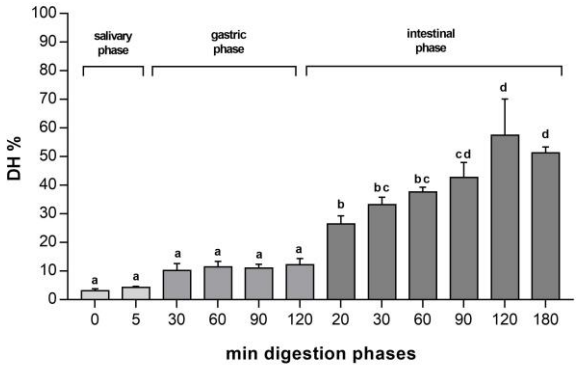

salivary phase
gastric phase
intestinal phase
DH %
min digestion phases
0
5
30
60
90
120
20
30
60
90
120
180
a
a
a
a
a
a
b
bc
bc
cd
d
d

Figure 2

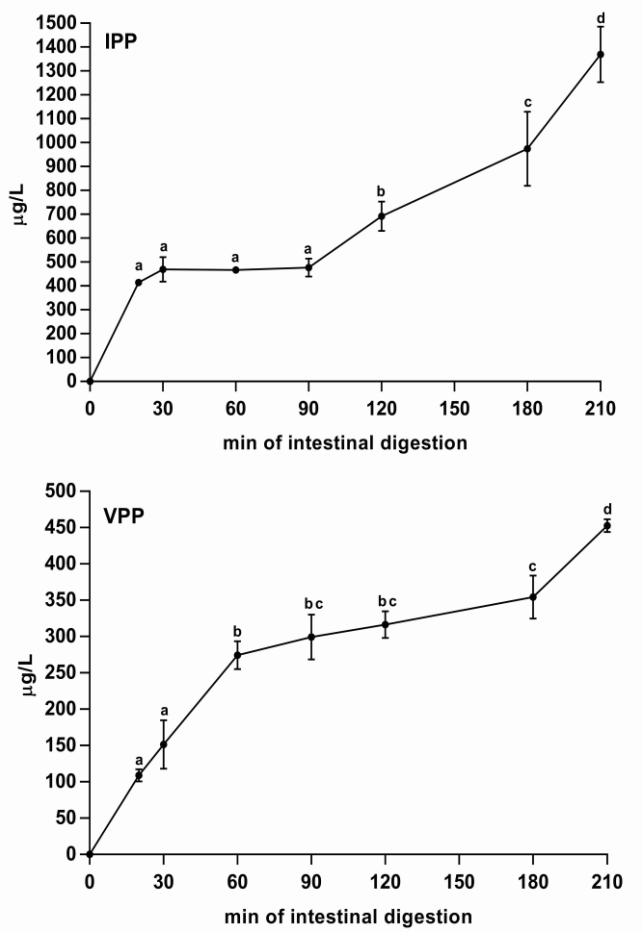

IPP
µg/L
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
a
a
a
a
b
c
d
0
30
60
90
120
150
180
210
min of intestinal digestion
VPP
µg/L
500
450
400
350
300
250
200
150
100
50
0
a
a
b
bc
bc
c
d
0
30
60
90
120
150
180
210
min of intestinal digestion

Figure 3

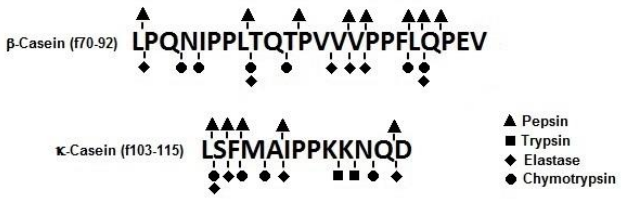
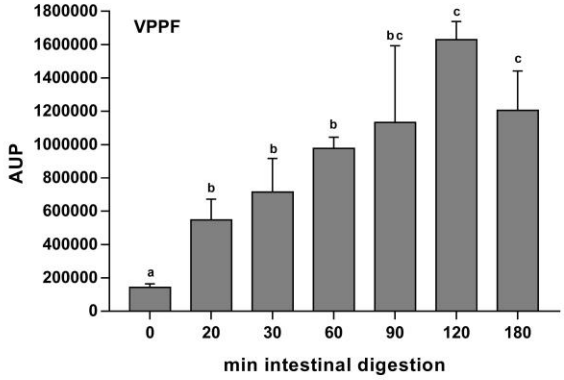
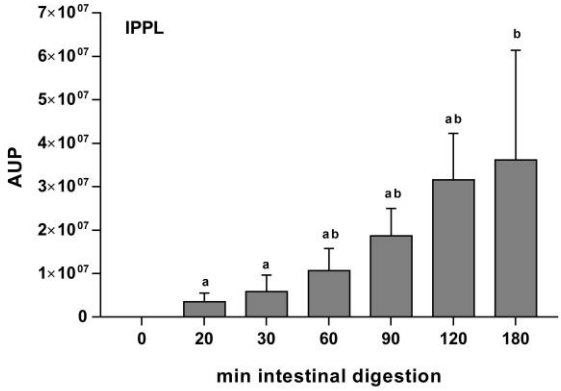
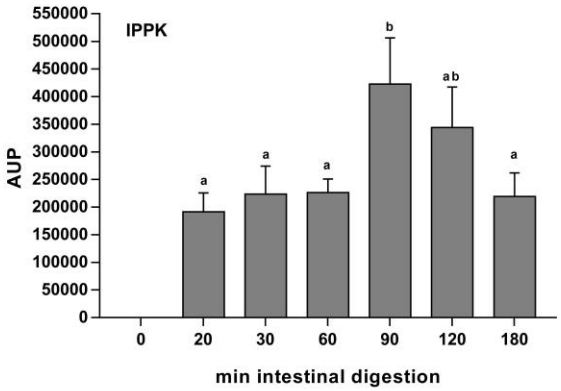

β-Casein (f70-92) LPQNIPPLTQTPVVVPPFLQPEV
κ-Casein (f103-115) LSFMAIPPKKNQD
Pepsin
Trypsin
Elastase
Chymotrypsin

Figure 4


VPPF
AUP
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
a
b
b
b
bc
c
c
0
20
30
60
90
120
180
min intestinal digestion


IPPL
AUP
7×10^07
6×10^07
5×10^07
4×10^07
3×10^07
2×10^07
1×10^07
0
a
a
ab
ab
ab
b
0
20
30
60
90
120
180
min intestinal digestion


IPPK
AUP
550000
500000
450000
400000
350000
300000
250000
200000
150000
100000
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0
a
a
a
b
ab
a
0
20
30
60
90
120
180
min intestinal digestion

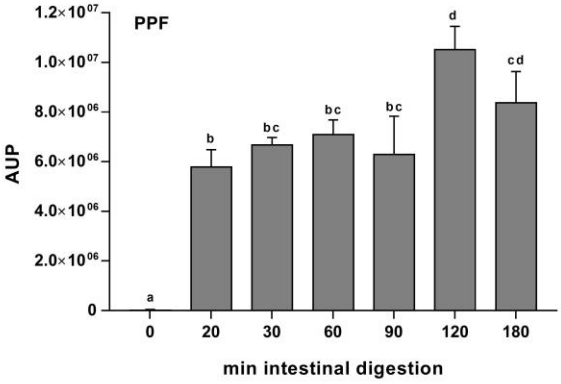
PPF
AUP
1.2×10^07
1.0×10^07
8.0×10^06
6.0×10^06
4.0×10^06
2.0×10^06
0
a
b
bc
bc
bc
d
cd
0
20
30
60
90
120
180
min intestinal digestion

Figure 5

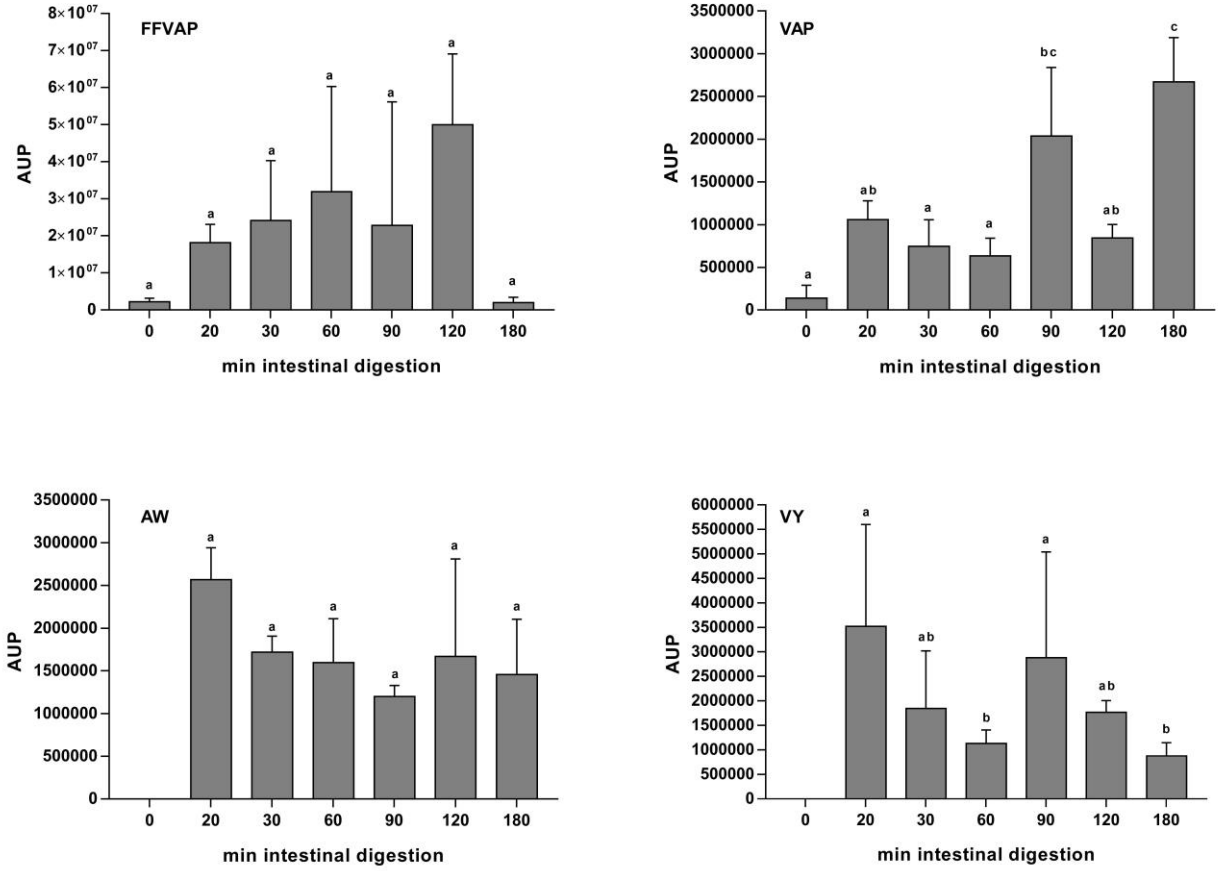
FFVAP
AUP
8×10^07
7×10^07
6×10^07
5×10^07
4×10^07
3×10^07
2×10^07
1×10^07
0
a a a a a a a
0 20 30 60 90 120 180
min intestinal digestion
VAP
AUP
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
a ab a a bc ab c
0 20 30 60 90 120 180
min intestinal digestion
AW
AUP
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
a a a a a a
0 20 30 60 90 120 180
min intestinal digestion
VY
AUP
6000000
5500000
5000000
4500000
4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
a ab b a ab b
0 20 30 60 90 120 180
min intestinal digestion

Table of content graphic