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Composition and properties of peptides that survive standardised in vitro gastro-pancreatic digestion of bovine milk

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ABSTRACT

There are conflicting results on the release and conservation of bioactive protein sequences during in vitro digestion. Only the peptides that are released and survive may exert their action. Peptides <3 kDa were determined by mass spectrometry after standardised in vitro gastro-pancreatic digestion of bovine milk. At a degree of digestion of 30.7%, 384 peptides were obtained from 2 to 25 residues in length. Five other peptides were recovered after reduction of disulphide bonds. Due to the presence of proline residues at the last or second last position at the C-terminus, a high number of peptides with ACE-inhibitory activity were obtained. Peptides with opioid, antimicrobial, immunomodulatory and antithrombotic activities were recovered, with many precursors and degradative products. Peptides with opioid and antimicrobial activities may be the result of evolutionary processes. The peptides obtained were similar to those released in the human jejunum.

1. Introduction

Several peptides have been isolated and identified after enzymatic hydrolysis of bovine milk proteins showing antihypertensive, antimicrobial, immunomodulatory, and opioid activities (Choi, Sabikhi, Hassan, & Anand, 2012; De Noni, 2008; Nagpal et al., 2011; Pihlanto-Leppälä, Rokka, & Korhonen, 1998; Ricci-Cabello, Herrera, & Artacho, 2012).

The active sequences present must be released from proteins during digestion to exert their actions at intestinal level or to be absorbed. In this respect, the intestine of newborn and young mammals is more permeable to peptides and antibodies then that of adults. The active peptides must survive in the digestive media for some period of time before being hydrolysed to amino acids.

There are contradictory results concerning the release and conservation of the bioactive peptides during digestion (Boutrou et al., 2013; Cieslinska, Kaminski, Kostyra, & Sienkiewicz-Szlapka, 2007; Picariello et al., 2010; Schmelzer et al., 2007). This is mainly due to the different methods and enzymes utilised in the in vitro digestion, which, in many cases, is far from physiological conditions. In some cases, the digestion of a single milk protein has been investigated. In other cases, Ca^{2+} and bile salts were not present, or enzymes with different specificity with respect to proteases of mammals were used (Nagpal et al., 2011; Ricci-Cabello et al., 2012).

There are few examples of digestion of milk proteins in vivo in humans (Boutrou et al., 2013; Chabance et al., 1998). Boutrou et al. (2013) administered caseins or whey proteins of bovine milk to healthy subjects and determined the sequence of all peptides recovered in the jejunum by mass spectrometry. They found that some bioactive peptides are conserved in this phase of digestion. It should be pointed out that, to be active at intestinal level, it is sufficient that the bioactive peptides are released from the protein in the duodenum, jejunum and upper ileum, as a result of gastro-pancreatic digestion.

The aim of this study was to report the entire set of peptides obtained from bovine milk digested with standardised in vitro gastro-pancreatic method of Minekus et al. (2014), to compare the results with those obtained in vivo by Boutrou et al. (2013).

Emphasis is given to the presence of known bioactive peptides, their precursors and products of their degradation. Only the peptides that are released and survive to gastro-pancreatic digestion

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may have physiological roles at least at intestinal level and may be the result of evolutionary processes.

The pool of peptides obtained will give new suggestions to implement the in vitro digestion of bovine milk proteins.

2. Materials and methods

2.1. Materials

Bile salts (a mixture of sodium cholate and sodium deoxycholate), dithiothreitol (DTT), porcine pepsin (≥2500 units mg solid^{-1}) and porcine pancreatin (4 × USP specifications) were supplied by Sigma (Milan, Italy). Amicon Ultra-4 centrifugal filter units (regenerated cellulose, nominal cut-off 3 kDa) were supplied by Millipore (Milan, Italy). Bovine skimmed milk was obtained from a local producer. The composition of unpasteurised skimmed milk used in this study was (in g 100 mL^{-1}): total protein, 3.1; caseins, 2.6; carbohydrates, 4.8; calcium, 0.12; fat, 0.05. Total solids, fat, ash, lactose, and nitrogen fractions were determined as reported by Tagliazucchi, Shamsia, and Conte (2016). Calcium was determined by titration with potassium permanganate after extraction and precipitation as oxalate. Chemicals and solvents for mass spectrometry analysis were from Carlo Erba (Milan, Italy).

2.2. In vitro gastro-pancreatic digestion

The standardised static in vitro digestion method of Minekus et al. (2014) was followed with minor modification for adaptation to milk. The oral phase of digestion was omitted since milk was considered as a liquid food and only the digestion of the proteins was investigated. To 100 mL of skimmed milk, simulated gastric fluid stock electrolyte solution was added and the pH was lowered to 2.5 with 1 mol L^{-1} HCl. Pepsin was then added to 2000 U mL^{-1} and the digestion mixture was incubated at 37 °C in a shaking bath for 2 h. The pH value of 2.5 was chosen since the pH, during the 2 h of peptic digestion, was maintained in the optimal interval of pepsin activity (pH 2–4), and the activity of gastric lipase was not taken into account. After 2 h, simulated intestinal fluid was added and the pH was increased to 7.5 with 20% Na_2CO_3 before adding 0.8 g L^{-1} pancreatin and 10 mmol L^{-1} of bile salts. The solution was incubated at 37 °C in a shaking bath for 2 h. At the end of the digestion, a fraction of the digest was immediately placed in ice and then diluted 1:5 with cold 0.1 mol L^{-1} potassium phosphate buffer, pH 7. Samples (3.5 mL each) were ultrafiltered using Amicon Ultra-4 centrifugal filter units, nominal cut-off 3 kDa, in a fixed-angle rotor at 7500 × *g* for 120 min at 4 °C. The permeate and retentate were kept at −20 °C until use. A control digestion, with the gastro-intestinal juices and enzymes and water in place of milk, was carried out to determine the effect of the digestive enzymes and their autolysis in the subsequent analysis. The digestions were carried out in triplicate.

The degree of hydrolysis was determined as described by Adler-Nissen (1979) for caseins. Degree of hydrolysis was calculated taking into account the data obtained in the control digestion (Tagliazucchi et al., 2016).

2.3. Reduction of permeate and retentate

Samples of permeate or retentate were reduced by the addition of 10 mmol L^{-1} DTT for 30 min at 56 °C in a thermomixer and ultrafiltered as reported above. The recovery of the peptides after reduction was determined.

2.4. Determination of peptides with nanoflow LC-ESI-QTOF MS analysis

The peptides, obtained before and after reduction, were determined with nanoflow liquid chromatography coupled with electrospray ionisation quadrupole/time-of-flight mass spectrometry (LC-ESI-QTOF MS). Nanoflow liquid chromatography-mass spectrometry (LC-MS) and tandem MS experiments were performed on a 1200 Series two-dimensional LC system coupled to a 6520 Accurate-Mass QTOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4 mm 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 consisted of (A) H_2O/acetonitrile/formic acid (96.9:3:0.1, by vol) and (B) acetonitrile/H_2O/formic acid (94.9:5:0.1, by vol). The sample (1 μL) was loaded onto the Chip enrichment column at a flow rate of 4 μL min^{-1} with a mobile phase consisting of 100% A using a G1376A capillary pump. A flush volume of 2 μL and a flush-out factor of 5 were used. After valve switching, a gradient elution was performed throughout the enrichment and analytical column at 500 nL min^{-1} using a G2226A nano pump. The gradient started at 0% B for 1 min then linearly ramped up to 90% B in 70 min. The mobile phase composition was maintained at 90% B for 15 min to wash both enrichment and analytical columns.

The mass spectrometer was tuned and calibrated according to the manufacturer's instructions in extended dynamic range (2 GHz) mode as reported by Dei Più et al. (2014).

For identification, MS/MS spectra were converted to .mgf files and were then searched against the Swiss-Prot database using the Protein Prospector protein identification software (http://prospector.ucsf.edu). The following parameters were considered: enzyme, none; peptide mass tolerance, ±40 ppm; fragment mass tolerance, ±0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modification permitted in a single peptide, 2. Only peptides with a best expected value lower than 0.05 that corresponded to $P < 0.01$ were considered. De novo peptide sequencing was performed using Pepnovo software (http://proteomics.ucsd.edu/ProteoSAFe/) and the same parameters as described above.

The assignment process was complemented and validated by the manual inspection of MS/MS spectra. The method used for peptide sequencing had some technological limitations. The first limitation is linked to the size of the peptides. The best range for peptide determination under our experimental conditions is between 100 and 5000 Da. A second limitation is regards the presence in the peptide sequence of post-translational modifications, which make peptide identification difficult. For example, the presence of disulphide bonds requires initial reduction for peptide identification. The presence of more than two phosphorylation sites resulted in a great increase in the search space and leads to a corresponding decrease in the confidence of identification (Steen & Mann, 2004). In dipeptides, the isomeric amino acids leucine (L) and isoleucine (I) are indistinguishable by LC-ESI-QTOF MS analysis. For this reason, when present in dipeptides, they are indicated by Lx where Lx may be L or I.

2.5. Identification of bioactive peptides

The biological activity of peptides was carried out through the Dziuba database updated 2015 (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php).