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Release of angiotensin converting enzyme-inhibitory peptides during in vitro gastro-intestinal digestion of camel milk

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ABSTRACT

Angiotensin-converting enzyme (ACE)-inhibitory peptides released from camel milk after simulated gastro-intestinal digestion were identified. The hydrolysis degree increased during digestion. The highest ACE-inhibitory activity was found in the post-pancreatic <3 kDa fraction. Peptides responsible for the biological activity were isolated by reversed-phase high-performance liquid chromatography and identified by mass spectrometry. Among the identified sequences, 17 were identical to known bioactive peptides with ACE-inhibitory activity. Based on previous structure–activity relationship studies, the sequence of some peptides allowed us to anticipate the presence of biological activities. The anti-hypertensive tripeptide isoleucine-proline-proline (IPP) was identified and quantified in digested camel milk. The amount of released IPP was 2.56 ± 0.15 mg L^{-1} of milk. For the first time, we showed that IPP is released during the gastro-intestinal digestion of camel milk κ-casein. This research provides the basis to increase the exploitation of the health benefits of camel milk.

1. Introduction

Previous research has recommended the potential use of camel milk as an alternative in case of cow milk allergy and in the manufacture of infant formulae (El-Agamy, Nawar, Shamsia, Awad, & Haenlein, 2009). In human milk as well as in camel milk, α-lactalbumin is the main whey protein, whereas β-lactoglobulin is absent. The latter protein is the principal responsible for the allergic reactions to cow milk in adult and children (El-Agamy et al., 2009).

Milk proteins possess unique properties and contain in their sequences encrypted peptides that show potential physiological properties and health benefits. These peptides are inactive when included in the protein sequence but can be released after hydrolysis (by enzymes or microorganisms) displaying their biological properties (Pihlanto, 2006). They can be released from both caseins (especially β-casein) and whey proteins and have been identified in milk of several species (Korhonen & Pihlanto, 2006).

The claimed biological activities of these peptides include antimicrobial, angiotensin-converting enzyme (ACE)-inhibitory, anti-hypertensive, antithrombotic and antioxidative activities (Nagpal et al., 2011). The main bioactive peptides studied are those with ACE-inhibitory activity (Phelan & Kerins, 2011). ACE is a dipeptidyl carboxypeptidase that catalyses, in vivo, the conversion of the plasmatic peptide angiotensin I into the potent vasoconstrictor angiotensin II. Inhibition of ACE plays an important role in blood pressure regulation and drugs that inhibit ACE are commonly prescribed for the treatment of hypertension or related cardiovascular diseases (Acharya, Sturrock, Riordan, & Ehlers, 2003). Several peptides that inhibit ACE were reported after enzymatic hydrolysis of milk proteins and after milk fermentation with Lactobacillus (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). Among ACE-inhibitory peptides derived from bovine caseins in milk fermented with *Lactobacillus*, valine-proline-proline [VPP; β-casein f(84–86)] and isoleucine-proline-proline [IPP; β-casein f(74–76)] have been the most studied (Solieri, Rutella, & Tagliazucchi, 2015). Other ACE-inhibitory peptides have been discovered in enzymatic hydrolysates of milk caseins and whey proteins (Phelan & Kerins, 2011). Digestive enzymes and combinations of different proteinases such as alcalase and thermolysin have been utilised to successfully generate bioactive peptides from various milk proteins. For example, two peptides derived from bovine α_{S1}-casein (RYLGY and AYFYPEL) after hydrolysis with pepsin, were able to reduce blood pressure in vivo when administered in spontaneously hypertensive rats (Contreras, Carrón,

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Montero, Ramos, & Recio, 2009). Three potent ACE-inhibitory peptides (LLF, LVRT, and LQKW) were purified from caprine β-lactoglobulin hydrolysed with thermolysin (Hernández-Ledesma, Recio, Ramos, & Amigo, 2002).

In a recent series of two papers, Picariello et al. (2010, 2013) studied the in vitro release of bioactive peptides after digestion of bovine milk caseins and whey proteins. They found that some ACE-inhibitory peptides (such as the sequence HLPLP from β-casein and the sequences GLDIQK and VLDTDYK from whey proteins) were released and stable under gastro-intestinal conditions and were able to across Caco-2 cells monolayers. ACE-inhibitory peptides (HLPLP and WSVPQPK) may be also released from human milk after in vitro gastro-intestinal digestion (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007). In addition, in vitro gastro-pancreatic digestion of donkey milk resulted in the release of a potent ACE-inhibitory peptide (VAPFPQPVVP) corresponding to the fragment f(176–185) of β-casein (Bidasolo, Ramos, & Gomez-Ruiz, 2012).

Only a few studies on the antioxidant and ACE-inhibitory activities of camel milk protein-derived peptides have been performed. Salami et al. (2011) and Moslehishad et al. (2013) found that camel milk proteins released ACE-inhibitory and antioxidant compounds after the treatment with digestive proteases or after fermentation with *Lactobacillus rhamnosus*. However, the sequence of bioactive compounds involved in the effect has not been revealed.

Caseins are, quantitatively, the most important proteins in camel milk. The sequence homology between bovine and camel milk caseins varies from 53% for α_{S1}-casein to 75% for β-casein. Although many bioactive sequences are not conserved in camel milk caseins, some portions capable of releasing peptides with ACE-inhibitory activities are present in the sequence of camel caseins (Kappeler, Farah, & Puhan, 1998).

In this study, therefore, we used an in vitro digestion procedure mimicking the chemical and physical condition of the gastro-intestinal tract to process skimmed camel milk. The digested sample was characterised for the ACE-inhibitory activity and then further separated with high-performance liquid chromatography (HPLC) and the different fractions characterised for their ACE-inhibitory activity. The fractions with the highest activity were then analysed with mass spectrometry (MS) with the aim to identify the bioactive peptides.

2. Materials and methods

2.1. Materials

Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine α-amylase, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (4 × USP), ACE from rabbit lung, mucin II and III, bovine serum albumin, 2,4,6-trinitrobenzenesulfonic acid (TNBS), sodium dodecyl sulphate (SDS), dithiothreitol (DTT), lysozyme and urea were supplied by Sigma (Milan, Italy). The tripeptide isoleucine-proline-proline (IPP; 95% purity) was synthesised by DBA (Milan, Italy). Amicon Ultra-4 regenerated cellulose 3 kDa were supplied by Millipore (Milan, Italy). The whole camel milk was obtained from farms at El-Alamin and Sidi-Barani areas around Alexandria (Egypt). All electrophoretic, HPLC and MS reagents were from Bio-Rad (Hercules, CA, USA.). All the other reagents were from Carlo Erba (Milan, Italy).

2.2. Chemical analysis and camel skimmed milk preparation

Whole camel milk was defatted, to obtain skimmed camel milk, by centrifugation at 2000 *g* for 20 min at room temperature. Skimmed milk sample was analysed for pH, total solids, fat and ash according to Ling (1963), lactose by phenol-sulphuric acid method (Marier & Baulet, 1959), nitrogen fractions, i.e., the total nitrogen, non-casein nitrogen by micro-Kjeldahl (Rowland, 1938).

2.3. In vitro gastro-intestinal digestion

For the in vitro digestion, the protocol, developed within the COST Action FA1005 and further validated for milk (Kopf-Bolanz et al., 2012), was followed.

Simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were prepared according to Kopf-Bolanz et al. (2012). SIF was prepared by mixing pancreatic (PF) and bile (BF) fluids. Skimmed camel milk (9 mL) was added to 12 mL of SSF containing 150 U mL^{-1} of porcine α-amylase and incubated for 5 min (oral phase). Gastric digestion was performed by adding 24 mL of SGF. The pH was adjusted to 2.0 with 6 mol L^{-1} HCl and supplemented with porcine pepsin (1115 U mL^{-1} of SGF). The gastric bolus was further incubated for 120 min (gastric phase). The intestinal digestion was carried out by adding to the gastric bolus 36 mL of SIF (24 mL of PF and 12 mL of BF), adjusting the pH to 7.0 and supplemented with pancreatin. The chyme was further incubated for 120 min (pancreatic phase). All incubations were performed at 37 °C on a rotating wheel (10 rpm). The digested samples were cooled on ice and immediately frozen at −80 °C for further analysis. The digestions were performed in triplicate.

A control digestion, which included only the gastro-intestinal juices and enzymes and water in place of milk, was carried out to consider the possible impact of the digestive enzymes in the subsequent analysis.

2.4. 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 (Adler-Nissen, 1979). The absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Orlando, FL, USA.).

The hydrolysis degree (DH) was calculated as reported in Equation (1):

$$DH = (h/h_{tot}) \times 100 \quad (1)$$

where h is the hydrolysis equivalent, defined as the concentration in milliequivalents per g of protein of α-amino groups formed at the different stages of the simulated digestion, and 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 (1979), the h_{tot} value was fixed at 8, which is the value calculated for caseins.

Hydrolysis degree data were calculated taking into account the data obtained in the control digestion.

2.5. SDS-polyacrylamide gel electrophoresis

Samples taken at different times of digestion were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis using 17% polyacrylamide separating gel, to evaluate the changes in protein profile during the hydrolysis (Helal, Tagliazucchi, Verzelloni, & Conte, 2014). Samples were diluted to same end dilutions in Laemmli buffer (0.05 mol L^{-1} Tris, pH 6.8, containing 2% SDS, 0.1 mol L^{-1} DTT, and 0.025% Bromophenol Blue). Vials were heated in boiling water for 4 min, and 5 μL of each sample (corresponding to 5 μg of undigested milk proteins) were loaded into the gel. The running conditions were 200 V, 60 mA, 1 h.