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Occurrence and fate of ACE-inhibitor peptides in cheeses and in their digestates following *in vitro* static gastrointestinal digestion

Milda Stuknytė, Stefano Cattaneo, Fabio Masotti, Ivano De Noni *

Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente, Università degli Studi di Milano, via G. Celoria 2, 20133 Milan, Italy

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ABSTRACT

The occurrence of the casein-derived angiotensin converting enzyme-inhibitor (ACE-I) peptides VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP and HLPLP were investigated in 12 different cheese samples by Ultra Performance Liquid Chromatography/High-Resolution Mass Spectrometry. The total amount of ACE-I peptides was in the range 0.87–331 mg kg^{-1}. VPP and IPP largely prevailed in almost all cheeses. Following *in vitro* static gastrointestinal digestion of Cheddar, Gorgonzola, Maasdam and Grana Padano cheeses, type and amount of ACE-I peptides changed, and only VPP, IPP, HLPLP and LHLPLP were detected in the intestinal digestates. The results evidenced that the degree of proteolysis itself cannot be regarded as a promoting or hindering factor for ACE-I peptide release during cheese digestion. Moreover, the data indicated that the ACE-I potential of cheeses cannot be inferred based on the type and amount of ACE-I peptides present in undigested samples.

1. Introduction

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1) is an important enzyme in the renin-angiotensin system, which plays a pivotal role in the regulation of blood pressure (BP; Matchar et al., 2008). It converts the peptide angiotensin I to the vasoconstrictor angiotensin II. Blocking production of angiotensin II with ACE inhibitors prevents constriction of blood vessels and lowers BP (Matchar et al., 2008). Several peptides represent competitive substrates for ACE, and their inhibiting activity is mainly modulated by a specific C-terminal sequence, which is required for inhibitors to bind to ACE. In this regard, ACE-inhibitor (ACE-I) peptides having Arg, Trp, Tyr, Phe or Pro residues in the three C-terminal positions have been reported as the most effective in enhancing binding to ACE (Gomez-Ruiz, Ramos, & Recio, 2004; Quirós et al., 2007). ACE-I peptides from casein (CN) have hydrophobic or positively charged amino acid residues, such as Pro, Lys or Arg, at the three C-terminal positions (FitzGerald, Murray, & Walsh, 2004; Murray & Fitzgerald, 2007). These findings attracted the scientific interest in the ACE-I potential of CN-derived peptides found in several cheeses (Gomez-Ruiz, Taborda, Amigo, Recio, & Ramos, 2006; Sagardia, Iloro, Elortza, & Bald, 2013). Usually peptides of 2–12 amino acids have been reported to be ACE-I, and for this reason the water-soluble cheese extracts (WSEs) after ultrafiltration (UF) at 3–10 kDa have been considered for evaluating ACE-I activity (López-Fandiño, Otte, & van Camp, 2006). Among ACE-I peptides identified in cheese and derived from bovine CN, VPP [β-CN f(84–86)] and IPP [β-CN f(74–76)] have been the most studied (Nakamura et al., 1995). Nonetheless, other ACE-I peptides have been discovered in enzymatic hydrolysates of α-, β- and κ-CN (FitzGerald et al., 2004; Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Jiang, Tian, Brodkorb, & Huo, 2010). Recently, two peptides derived from bovine α_{S1}-CN, with sequences RYLGY [α_{S1}-CN f(90–94)] and AYFYPEL [α_{S1}-CN f(143–149)], have demonstrated potent BP reducing effects in spontaneously hypertensive rats (SHR) (Contreras, Carron, Montero, Ramos, & Recio, 2009). In addition, it was reported that peptides RYLGY and AYFYPEL could reduce BP in hypertensive humans (Contreras et al., 2009). Two other peptides, with sequences LHLPLP [β-CN f(133–138)] and HLPLP [β-CN f(134–138)], showed antihypertensive effect in SHR (Miguel, Contreras, Recio, & Aleixandre, 2009; Quirós et al., 2007).

Gastrointestinal digestion may cause the degradation of bioactive peptides or lead to the formation of active fragments from inactive or less active precursors. Most of the studies concerning the bioavailability of ACE-I peptides have been aimed at *in vitro*, evaluating their resistance to gastrointestinal digestion (Contreras, Sancho, Recio, & Mills, 2012; Kopf-Bolanz et al., 2012; Quirós, Davalos, Lasuncion, Amigo, & Recio, 2008). For instance, peptides IPP and VPP have been shown to be *in vitro* resistant to single digestive peptidases (Ohsawa et al., 2008). Following *in vitro* digestion with pepsin and Corolase®, the peptides RYLGY,

* Corresponding author. Tel.: +39 0250316680; fax: +39 0250316672.
E-mail address: ivano.denoni@unimi.it (I. De Noni).

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AYFYPEL and LHLPLP were (partially) resistant to proteolysis (Quirós, Contreras, Ramos, Amigo, & Recio, 2009; Quirós et al., 2008). Bioactive peptides may be also degraded in the gastrointestinal tract and by brush border peptidases. *In vitro* experiments demonstrated that RYLGY and AYFYPEL are absorbed partially intact through cell monolayers (Contreras et al., 2012). Similar findings were reported on intestinal absorption of VPP, IPP and HLPLP (Foltz et al., 2007; Quirós et al., 2008; Satake et al., 2002).

According to already published results, no data are available on the actual levels of RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP and HLPLP in many cheeses from bovine milk and in their digestates. Moreover, no study has yet been performed on the resistance or occurrence of these peptides during *in vitro* static gastrointestinal digestion (SGID) of cheese. Therefore, the main objective of the present work was to evaluate for the first time the occurrence of these ACE-I peptides, as well as that of VPP and IPP, in cheeses with different degree of proteolysis, and to assess the stability of these peptides during *in vitro* SGID. To this purpose, we developed an Ultra Performance Liquid Chromatography/High Resolution Mass Spectrometry (UPLC/HR-MS) method for targeted ACE-I peptide detection in (digested) cheese samples. The effect of SGID on the occurrence and fate of ACE-I peptides was assessed by an *in vitro* static protocol.

2. Materials and methods

2.1. Synthetic ACE-I peptides

Synthetic ACE-I peptides VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP and HLPLP were purchased from Genscript (Piscataway, NJ, USA). Peptides with the same sequence have been reported to derive from bovine milk CN and to possess ACE-I activity in *in vitro* or *in vivo* studies (Table 1).

2.2. Cheese samples

Cheese samples considered in the present work were: Brie, Caprino, Cheddar, Emmental Protected Designation of Origin (PDO), Fontina PDO, Gorgonzola PDO, Gouda, Grana Padano PDO, Maasdam, Provolone PDO, Provolone piccante PDO and Taleggio PDO. All samples were collected at the Italian market. The duration of cheese ripening is presented in Table 2.

2.3. Assessment of cheese proteolysis

Total nitrogen (TN) content of cheese samples was determined according to the International standard ISO 8968-1 (2014). Proteolysis was measured by the determination of nitrogenous fractions of cheese samples according to the International standard ISO 27871 (2011). In detail, the soluble nitrogen mass fraction at pH 4.4 (SN), the soluble nitrogen contents in (120 g l^{-1}) trichloroacetic acid (TCA–SN) and in (5 g l^{-1}) phosphotungstic acid (PTA–SN) were determined. Capillary zone electrophoresis (CZE) was used to ascertain the CN and peptide profile in cheese samples. For this purpose, a Beckman System MDQ (Beckman Instruments, Fullerton, CA, USA) was adopted. Separations were performed according Masotti, Hogenboom, Rosi, De Noni, and Pellegrino (2010) and using an hydrophilically coated fused-silica capillary column (DB-WAX 126-7012, 50 mm i.d., 0.05 mm coating; J & W Scientific, Agilent Technologies, Santa Clara, CA, USA). The capillary column was cut manually and low-flame window burning was used to remove the polyimide coating. Each cheese sample was subjected to three replicate analyses and mean values are presented.

Table 1
IC_{50}-values of the ACE-I peptides studied in the present work.

Sequence	Bovine casein source	IC_{50} (μM)	References
VPP	β-CN f(84–86)	9	Nakamura et al. (1995)
IPP	β-CN f(74–76)	5	Nakamura et al. (1995)
RYLG	α_{S1}-CN f(90–93)	225	Contreras, Sanchez, Sevilla, and Recio (2013)
RYLGY	α_{S1}-CN f(90–94)	0.71	Contreras et al. (2009, 2013)
AYFYPE	α_{S1}-CN f(143–148)	261	Contreras et al. (2013)
AYFYPEL	α_{S1}-CN f(143–149)	6.58	Contreras et al. (2009, 2013)
HLPLP	β-CN f(134–138)	21	Hernández-Ledesma, Quirós, Amigo, and Recio (2007) and Quirós et al. (2009, 2008)
LHLPLP	β-CN f(133–138)	5.5	Quirós et al. (2007)
		3.7	Quirós et al. (2009)

Table 2
Levels of total nitrogen (TN), soluble nitrogen (SN), soluble nitrogen in trichloroacetic acid (TCA–SN) and soluble nitrogen in phosphotungstic acid (PTA–SN) of the analysed cheeses samples.

Sample	TN (%)	SN (%)	TCA–SN (% SN)	PTA–SN (% SN)
Brie (1)	2.65 ± 0.03	0.68 ± 0.03	50.0	13.8
Caprino (0.5)	2.22 ± 0.01	0.22 ± 0.01	42.9	18.3
Cheddar (4)	3.87 ± 0.04	1.23 ± 0.04	81.8	49.9
Emmental (4)	4.37 ± 0.03	1.27 ± 0.03	58.0	28.9
Fontina (3)	4.07 ± 0.03	0.67 ± 0.01	48.3	18.0
Gorgonzola (2)	2.89 ± 0.01	1.90 ± 0.04	83.0	34.6
Gouda (2.5)	3.45 ± 0.04	0.61 ± 0.03	59.8	21.6
Grana Padano (11)	5.29 ± 0.05	1.25 ± 0.03	85.7	59.3
Maasdam (2)	4.09 ± 0.04	1.04 ± 0.02	49.3	22.1
Provolone (2)	3.90 ± 0.03	0.70 ± 0.03	75.2	44.5
Provolone piccante (4)	3.88 ± 0.02	0.79 ± 0.02	79.5	44.2
Taleggio (2)	2.91 ± 0.03	0.69 ± 0.02	48.1	15.6

Months of cheese ripening are indicated in brackets. Values are presented as means ± SD (n = 3).

2.4. Preparation of water-soluble cheese extracts

Water-soluble cheese extracts were obtained according Bütikofer, Meyer, Sieber, and Wechsler (2007) with slight modifications. Five grams of cheese were homogenised in 10 ml of 0.1 N HCl for 1 min at 17,000 rpm min^{-1} using an Ultra-turrax T25 apparatus (IKA-Labortechnik, Staufen, Germany). The mixture was kept at 40 °C for 1 h under gentle shaking in a Multi Bio RS-24 rotator (Biosan, Riga, Latvia). The sample was centrifuged at 10,000*g* for 15 min at 10 °C. To remove high molecular mass peptides the WSE was ultrafiltered through Omega modified polyethersulfone UF membrane (cut-off 3 kDa) in a Nanosep Advance device (Pall, Port Washington, NY, USA) centrifuging 14,000*g* for 90 min. The permeate was collected and stored at −40 °C until UPLC/HR-MS.

2.5. In vitro static gastrointestinal digestion

Digestions of cheeses were carried out using an *in vitro* SGID protocol. In detail, simulated salivary (SSF), simulated gastric (SGF) and simulated duodenal (SDF) fluids were prepared according Kopf-Bolanz et al. (2012) and Versantvoort, Oomen, van de Kamp, Rompelberg, and Sips (2005). Cheese samples (2.5 g) were ground in a mincer in presence of 5 ml of SSF at pH 7.0 for 2 min to reproduce mastication. The derived bolus was mixed with 5 ml of SGF supplemented with porcine pepsin (1000 U ml^{-1} of SGF). The gastric phase digestion was performed at 37 °C for 2 h at pH 3.0 (adjusted with 1 N HCl). Afterwards, 10 ml of SDF and bile salts (10 mM, Sigma–Aldrich, Milan, Italy) were added to the digestate. Enzymes for intestinal digestion were porcine trypsin (100 U ml^{-1} SDF), bovine chymotrypsin (50 U ml^{-1} SDF), porcine intestinal lipase (2000 U ml^{-1} SDF) and co-lipase (molar ratio