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Release of angiotensin converting enzyme-inhibitor peptides during in vitro gastrointestinal digestion of Parmigiano Reggiano PDO cheese and their absorption through an in vitro model of intestinal epithelium

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ABSTRACT

The occurrence of 8 bovine casein-derived peptides (VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP, and HLPLP) reported as angiotensin converting enzyme-inhibitors (ACE-I) was investigated in the 3-kDa ultrafiltered water-soluble extract (WSE) of Parmigiano Reggiano (PR) cheese samples by ultra-performance liquid chromatography coupled to high-resolution mass spectrometry via an electrospray ionization source. Only VPP, IPP, LHLPLP, and HLPLP were revealed in the WSE, and their total amount was in the range of 8.46 to 21.55 mg/kg of cheese. Following in vitro static gastrointestinal digestion, the same ACE-I peptides along with the newly formed AYFYPEL and AYFYPE were found in the 3 kDa WSE of PR digestates. Digestates presented high amounts (1,880–3,053 mg/kg) of LHLPLP, whereas the remaining peptides accounted for 69.24 to 82.82 mg/kg. The half-maximal inhibitory concentration (IC_{50}) values decreased from 7.92 ± 2.08 in undigested cheese to 3.20 ± 1.69 after in vitro gastrointestinal digestion. The 3-kDa WSE of digested cheeses were used to study the transport of the 8 ACE-I peptides across the monolayers of the Caco-2 cell culture grown on a semipermeable membrane of the transwells. After 1 h of incubation, 649.20 ± 148.85 mg/kg of LHLPLP remained in the apical compartment, whereas VPP, IPP, AYFYPEL, AYFYPE, and HLPLP accounted in total for less than 36.78 mg/kg. On average, 0.6% of LHLPLP initially present in the digestates added to the apical compartment were transported intact to the basolateral chamber after the same incubation time. Higher transport rate (2.9%) was ascertained for the peptide HLPLP. No other intact ACE-I peptides were revealed in the basolateral compartment. For the first time, these results demonstrated that the ACE-I peptides HLPLP and LHLPLP present in the in vitro digestates of PR cheese are partially absorbed through an in vitro model of human intestinal epithelium.

Key words: Parmigiano Reggiano, angiotensin converting enzyme-inhibitor peptides, in vitro gastrointestinal digestion, in vitro intestinal absorption, ultra-performance liquid chromatography coupled to high-resolution mass spectrometry via an electrospray ionization source

INTRODUCTION

Parmigiano Reggiano (**PR**) is one of the best-known and most exported Italian cheeses all over the world (Progetto Si P-R, 2015). It is an extra-hard, long-ripened cheese produced from raw cow milk after natural partial creaming and is registered as a Protected Designation of Origin cheese (Council Regulation EC No. 510, 2006). Parmigiano Reggiano cheese is produced exclusively in the provinces of Parma, Reggio Emilia, Modena, and parts of the provinces of Mantova and Bologna. The entire cheese-making process is rigorously regulated; for instance, only natural whey starter (**NWS**) resulting from previous cheese making can be added to starting raw milk. Microorganisms present in the natural whey starter are mainly represented by thermophilic starter lactic acid bacteria selected during cooking (54–55°C) of the curd and subsequent incubation of the rennet whey from cheese making. These starter lactic acid bacteria along with nonstarter lactic acid bacteria (**NSLAB**) naturally present in raw milk strongly contribute to CN breakdown as proteolysis represents the most intense biochemical process during PR maturation, which lasts not less than one year. As a consequence, the proteolytic activities of starter lactic acid bacteria and nonstarter lactic acid bacteria generate a wide array of (oligo)peptides, some of which

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are or contain potential bioactive sequences (Battistotti and Corradini, 1993).

Angiotensin I-converting enzyme (**ACE**; EC 3.4.15.1) belongs to the renin-angiotensin system acting in the regulation of blood pressure (Matchar et al., 2008). Some peptides, which possess a specific C-terminal sequence, are capable of binding to ACE, and based on this, they represent competitive substrates for the above-mentioned enzyme (Gomez-Ruiz et al., 2004; Quirós et al., 2007). Some peptides released during cheese ripening share these features and, therefore, they have been studied and assessed for their in vitro ACE-inhibition potential (FitzGerald et al., 2004; Gomez-Ruiz et al., 2006; Sagardia et al., 2013). In this regard, because the water-soluble extract (**WSE**) of cheeses contains most of the peptides, it is usually assessed for inhibition of ACE activity by measuring the half-maximal inhibitory concentration ($\mathbf{IC_{50}}$) value in vitro. This last represents the concentration of bioactive peptides able to inhibit the ACE activity by 50%. Most of the ACE-inhibitor (**ACE-I**) peptides are short sequences arising from breakdown of bovine CN, and they are usually isolated by UF of the cheese WSE at 3 to 10 kDa (López-Fandiño et al., 2006; Stuknytė et al., 2015). The peptides VPP [β-CN f(84–86)] and IPP [β-CN f(74–76)] have been the most investigated (Nakamura et al., 1995; Bernard et al., 2005), whereas other ACE-I peptides have been discovered in enzymatic hydrolysates of bovine α-, β-, and κ-CN (FitzGerald et al., 2004; Jiang et al., 2010; Hernández-Ledesma et al., 2014). More recently, Contreras et al. (2009) showed that RYLGY [α_{S1}-CN f(90–94)] and AYFYPEL [α_{S1}-CN f(143–149)] potently reduce blood pressure when they are administered to hypertensive humans or spontaneously hypertensive rats. In addition, Quirós et al. (2007) and Miguel et al. (2009) ascertained the hypotensive effect of the peptides LHLPLP [β-CN f(133–138)] and HLPLP [β-CN f(134–138)].

To establish any potential in vivo antihypertensive effect, the fate of ACE-I peptides during gastrointestinal digestion (**GID**) as well as their fate and absorption through the intestinal epithelium have to be preliminary evaluated. Indeed, GID of cheese may lead to either degradation or formation of these peptides. Several protocols are nowadays available for in vitro GID (Quirós et al., 2008; Contreras et al., 2012; Kopf-Bolanz et al., 2012). Nonetheless, only the protocol recently proposed by Minekus et al. (2014) has been internationally accepted as a physiologically relevant model for in vitro GID studies. The transepithelial transport of peptides as well as their degradation at the intestinal tract by cellular peptidases are studied in vitro using model cell cultures. In this regard, Caco-2 cells from human colorectal adenocarcinoma are considered a reliable in vitro model of intestinal epithelium because they express several morphological and functional properties characteristic of small bowel enterocytes (Sambuy et al., 2005). For instance, at confluence, cells polarize and acquire an apical brush border with microvilli. Moreover, they express enzymatic activities typical of enterocytes (e.g., aminopeptidase N and dipeptidylpeptidase IV).

To date, no studies have been performed on the presence of ACE-I peptides in PR cheese or on the fate of these peptides during in vitro GID of this cheese. Moreover, no studies dealt with the transepithelial transport of the ACE-I peptides present in PR digestates. On these bases, the first objective of the present work was to evaluate the occurrence of 8 ACE-I peptides in PR samples and to assess the peptide content after in vitro GID of the cheese. Furthermore, the present work aimed to investigate the ACE-I potential of the 3-kDa ultrafiltered fractions from WSE of both undigested and digested PR samples. Finally, the transepithelial transport of the ACE-I peptides present in PR digestates was studied using Caco-2 monolayers in vitro.

MATERIALS AND METHODS

Parmigiano Reggiano Cheese Samples

Six samples of 12-mo-aged PR cheese were obtained from 6 different dairies located in the provinces of Mantova, Parma, and Reggio Emilia. Cheese samples were vacuum-wrapped and stored for a maximum of 5 d at 4°C before analysis.

Extraction of 3 kDa of WSE and In Vitro Static GID of PR Cheese

The WSE of PR cheese samples were obtained according to the procedure described by Rizzello et al. (2005) with some modifications. Briefly, minced cheese was suspended in deionized water and acidified to pH 4.4 with 1 *M* HCl to precipitate CN. The supernatant was filtered (Whatman grade 2, GE Healthcare, Little Chalfont, UK), centrifuged at 3,000 × *g* for 30 min at 4°C. The 3-kDa permeate of WSE was obtained using an Amicon Ultra 3 kDa UF system (Amicon, Millipore, Bedford, MA) and then stored at –80°C until use.

Digestions of cheeses were carried out using the in vitro GID protocol reported by Minekus et al. (2014). Cheese samples (2.5 g) were ground in a mincer in the presence of 5 mL of simulated salivary fluid (**SSF**) at pH 7.0 for 2 min to reproduce mastication. The derived bolus was mixed with 5 mL of simulated gastric fluid (**SGF**) supplemented with porcine pepsin (1,000 U/mL of SGF). The gastric phase digestion was performed

at 37°C for 2 h at pH 3.0 (adjusted with 1 *N* HCl). Afterward, 10 mL of simulated duodenal fluid (**SDF**) and bile salts (10 m*M*, Sigma-Aldrich, St. Louis, MO) were added to the digestate. Enzymes for intestinal digestion were porcine trypsin (100 U/mL of SDF), bovine chymotrypsin (50 U/mL of SDF), porcine intestinal lipase (2000 U/mL of SDF), and colipase (molar ratio lipase/colipase: 1/2). The intestinal phase digestion was performed at 37°C for 2 h at pH 7.0, and it was stopped by adding the protease inhibitor AESFB (Roche, Mannheim, Germany) to give a 1 m*M* final concentration. The digestates were immediately frozen at 40°C and freeze-dried. Each cheese sample was submitted to 2 replicate digestions on the same day. The 3-kDa permeate of digestates was extracted using the above-mentioned UF system, and then stored at −80°C until use. The bile salts and the enzymes were purchased from Sigma-Aldrich. All the 3 kDa permeates of WSE were analyzed to assess the concentration of ACE-I peptides and to determine the ACE-inhibitory activity.

Determination of ACE-Inhibitory Activity of WSE of Undigested and In Vitro Digested PR Cheeses

The concentration of peptides per milliliter of 3-kDa WSE of PR cheeses and their ACE-inhibitory activity were measured as previously described by Bernabucci et al. (2014). The concentration of peptides in the WSE was determined by a fluorimetric method (FluoroProfile Protein Quantification Kit, Sigma-Aldrich) using a commercial kit according to the manufacturer's instructions. The peptide determination was carried out in quadruplicate and fluorescence signal was read using a Multimode Detector DTX 880 (Beckman Coulter Inc., Fullerton, CA).

The ACE-inhibitory activity of WSE was measured by a colorimetric method (ACE kit-WST, Dojindo Inc., Kumamoto, Japan) according to the manufacturer's instructions. The WSE of digested and nondigested samples were tested in triplicate. Absorbance was read using a Sunrise Plate Reader (Tecan Trading AG, Mannedorf, Switzerland) and IC_{50} (the concentration needed to achieve the 50% inhibition of the ACE activity in the in vitro assay) was expressed as micrograms of peptides per milliliter of 3 kDa WSE.

Caco-2 Cell Cultures, Cytotoxicity Study, and Transepithelial Transport Experiments

Caco-2 cells originating from clone C2BBe1 [American Type Culture Collection (**ATCC**), CRL-2102, Manassas, VA] were maintained at 37°C in 5% CO_2 atmosphere, in complete Dulbecco's modified Eagle's medium (**DMEM**; ATCC) supplemented with 0.01 mg/mL of human transferrin (Sigma-Aldrich), 100 U/L of penicillin, 100 μg/L of streptomycin (Sigma Aldrich), and 10% (vol/vol) heat-inactivated fetal calf serum (ATCC). Cells were maintained in complete DMEM in a 75-cm^2 flask (Corning, NY) split at 50% of confluence and used for the differentiation and for the transport experiment between passages 59 to 60. For the differentiation experiments, cells were seeded onto the polyethylene-terephthalate filter inserts with 0.4-μm pore diameter and 1.12 cm^2 area (Corning) at a density of 3×10^5 cells/cm^2 and maintained for 21 d in a complete DMEM. The medium was changed 3 times a week, both for maintenance and differentiation.

Neutral red (**NR**; Sigma-Aldrich) test was performed as described by Sakai et al. (1998) with some modifications to test the cytotoxicity of digested PR samples. To this purpose, the freeze-dried 3-kDa WSE of digestates were solubilized in Hanks' balanced salt solution (**HBSS**; Sigma-Aldrich) at 0.1, 0.2, 0.4, and 0.8 mg/mL of total peptides and were used to establish the noncytotoxic concentration. In detail, at the end of each transport experiment, cell monolayers were exposed to 0.5 mL of HBSS containing 50 μg/mL of NR both in apical and basolateral chambers, and incubated overnight at 37°C. Then, Caco-2 monolayers were fixed with a formaldehyde solution (1%, vol/vol) containing $CaCl_2$ (1%, wt/vol) for 2 min at 25°C. After fixation, NR was extracted from each cell monolayer using 1 mL of aqueous ethanol (50%, vol/vol) solution of acetic acid (1%, vol/vol) overnight at 25°C. Finally, 100 μL of the supernatant were used for spectrophotometric measurements at a wavelength of 540 nm. On the basis of control experiments, 3 kDa WSE of PR digestates at the concentration of 0.4 mg/mL of HBSS were finally chosen for the transport experiments.

The integrity of the Caco-2 monolayers was checked by monitoring the transepithelial electrical resistance (**TEER**) with a Millicell-ERS device (Millipore, Darmstadt, Germany) before and after the transport experiments. Prior to transport experiment, the Caco-2 monolayers were rinsed twice with HBSS (pH 7.4) for 20 min at 37°C to stabilize the physiological parameters. Caco-2 monolayers were then incubated with 0.4 mL of HBSS (pH 6.5) containing the test sample in an apical compartment, and with 1.2 mL of HBSS (pH 7.4) in the basolateral compartment. The experiment lasted 1 h at 37°C in a 5% CO_2 atmosphere. At the end of the transport experiment the donor solution in the apical side and the receiving solution in the basolateral compartment were removed and quickly frozen at −80°C until analysis by ultra-performance liquid chromatography coupled to high-resolution mass spectrometry via an electrospray ionization source (**UPLC/ESI-HRMS**).

The ACE-I peptides were quantified to evaluate their apparent permeability coefficients (P_{app}) across the Caco-2 monolayers. The P_{app} was calculated according to the following equation:

$$P_{app} = 1/(A \cdot C_0) \cdot dQ/dT,$$

where A is the surface area of the Caco-2 monolayer (1.12 cm^2), C_0 is the initial ACE-I peptide concentration in the donor apical chamber (μmol/mL), and dQ/dT is the amount of ACE-I peptides measured in the acceptor basolateral chamber as a function of time (μmol/s).

Determination of ACE-I Peptides by UPLC/ESI-HRMS

The UPLC/ESI-HRMS analysis reported in Stuknytė et al. (2015) was used to identify and quantify the studied ACE-I peptides in 3-kDa WSE of undigested and digested PR samples, and in both the apical and basolateral compartments of the transwells during in vitro transport experiments through Caco-2 cell monolayers.

Synthetic ACE-I peptides VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP, and HLPLP were purchased from GenScript (Piscataway, NJ).

Statistical Analysis

Data were analyzed by one-way ANOVA. The differences were analyzed by the *t*-test, and the significances were set at a value of $P < 0.05$. The analysis was carried out using the Statistica 7.0 software (Stat Soft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Occurrence of ACE-I Peptides in 3 kDa WSE of Undigested PR Samples

The peptides VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP, and HLPLP have been reported to derive from bovine milk CN and to possess potent ACE-I activity in in vitro or in vivo studies (Nakamura et al., 1995; FitzGerald et al., 2004; Quirós et al., 2007; Contreras et al., 2009; Miguel et al., 2009; Jiang et al., 2010; Hernández-Ledesma et al., 2014). For this reason, in the present study we chose them as the target peptides to be searched and quantified using UPLC/ESI-HRMS. Despite several studies focused on the (oligo) peptide fraction of PR cheese, none of them investigated the presence and quantified the ACE-I peptides VPP, IPP, RYLGY, RYLG, AYFYPEL, AYFYPE, LHLPLP, and HLPLP in both PR cheese and in its digestates. In the present study, the total amount of these ACE-I peptides in undigested PR samples was in the range 8.46 to 21.55 mg/kg, and it was attributable only to the presence of VPP, IPP, LHLPLP, and HLPLP (Table 1). The portion of ACE-I peptides in the total peptide content of undigested WSE was 0.10 to 0.38% (Table 1).

Type and amount of ACE-I peptides found in the 6 samples of 12-mo-aged PR overlapped those previously identified in an 11-mo-aged Grana Padano sample (Stuknytė et al., 2015). Grana Padano and PR cheeses share common manufacturing process and similar proteolytic phenomena during ripening, and comparable breakdown of CN has been demonstrated at the same time of curing (Battistotti and Corradini, 1993). In general, the content of ACE-I peptides in the PR samples was low in comparison with levels usually found in short- to medium-ripened undigested cheeses (Pripp et al., 2006; Hayes et al., 2007; Stuknytė et al., 2015), the WSE of which are reported to possess the most potent ACE-I activities (Sieber et al., 2010). This could be explained by long maturation of PR cheese and higher extent of proteolysis Indeed, Sieber et al. (2010) and Pripp et al. (2006) reported ACE-I activity of cheeses to increase until a certain level of maturation and subsequently to decrease.

Release of ACE-I Peptides During In Vitro GID of PR Cheese Samples

The sequences of the ACE-I peptides revealed in the 3-kDa WSE of undigested PR samples are potentially sensitive to the action of different gastrointestinal enzymes (Parrot et al., 2003; Stuknytė et al., 2015). At the same time, gastrointestinal enzymes could release ACE-I peptides from precursor inactive oligopeptides generated from CN breakdown during PR ripening. Based on this, we evaluated the content of these peptides after in vitro GID of PR cheeses. The total amount of ACE-I peptides in digested PR samples was in the range of 1,959.33 to 3,122.52 mg/kg, and the portion of ACE-I peptides in the total peptide content of digested WSE was 13.68 to 21.81% (Table 1).

At the end of GID, we found relevant amounts of LHLPLP (2,483.95 ± 460.15 mg/kg) and HLPLP (58.97 ± 11.30 mg/kg; Table 1). Other detected peptides included the newly formed AYFYPEL (6.46 ± 4.71 mg/kg) and AYFYPE (7.34 ± 6.06 mg/kg), whereas we detected VPP and IPP at 3.97 ± 1.63 mg/kg and 2.32 ± 1.06 mg/kg, respectively. Using the same protocol for in vitro digestion of different cheeses, Stuknytė et al. (2015) revealed HLPLP and LHLPLP to occur only in digestate of an 11-mo-aged Grana Padano cheese. Indeed, in the same study, the release of ACE-I peptides during in vitro GID was found to be strongly cheese-type dependent. In this regard, a profile of ACE-

Table 1. Levels (mg/kg) of angiotensin converting enzyme-inhibitor (ACE-I) peptides revealed in the 3 kDa water-soluble extract of undigested and in vitro digested Parmigiano Reggiano cheese samples

Cheese sample		VPP	IPP	RYLG	RYLGY	AYFYPE	HLPLP	AYFYPEL	LHLPLP	Total ACE-I peptides	Total peptides	Portion (%) of ACE-I on total peptides
1–6	Undigested											
	Mean	4.67	3.08				1.25[a]		4.93[a]	13.92	7.238[a]	0.20[a]
	SD	3.23	1.75				0.11		2.00	5.87	1,397	0.10
1–6	Digested											
	Mean	3.97	2.32			7.34	58.97[b]	6.46	2,483.95[b]	2,561.78	14.293[b]	18.56[b]
	SD	1.63	1.06			6.06	11.30	4.71	460.15	459.30	3,663	2.94

[a,b]Means within a column with different superscripts differ ($P < 0.05$).

I peptides rich in HLPLP and LHLPLP was almost peculiar to PR and Grana Padano cheeses. In the same manner, this pattern of ACE-I peptides may be fundamental to argue the ACE-inhibitory potential of the 2 (digested) cheeses. Indeed, the peptides LHLPLP and HLPLP have been reported to potently reduce blood pressure when administered to hypertensive humans or spontaneously hypertensive rats (Quirós et al., 2007; Miguel et al., 2009).

ACE-Inhibitory Activity of 3 kDa WSE from Undigested and In Vitro Digested PR Samples

The ACE inhibitory activity of the WSE from undigested and in vitro digested PR samples showed a mean IC_{50} value of 7.92 ± 2.08 (μg of peptides/mL) and 3.20 ± 1.69 (μg of peptides/mL), respectively (Supplemental Table S1; http://dx.doi.org/10.3168/jds.2015-9801). These values are in accordance with the results previously reported by Bernabucci et al. (2014), and they are almost similar to those shown by several individual ACE-I peptides isolated from the WSE of different cheese varieties (Nakamura et al., 1995; Ohsawa et al., 2008; Sieber et al., 2010). Cheese digestates exhibited greater ($P < 0.05$) ACE-inhibitory activity, which might be associated with the increased content of ACE-I peptides formed during in vitro GID, including those studied in the present study. To the best of our knowledge, these are the first findings concerning the study and comparison of the ACE-inhibitory activity of WSE of both undigested and digested PR cheese. Moreover, the same results clearly demonstrate that a reliable evaluation of the potential hypotensive effect of any cheese should be assessed only after (in vitro) digestion.

Absorption of ACE-I Peptides Through the Caco-2 Monolayer In Vitro

To evaluate the Caco-2 cell viability after administration of the 3-kDa WSE of digested PR samples, we conducted the NR test before transport experiments of ACE-I peptides. The WSE of digestates did not affect the NR uptake because it was not different ($P > 0.05$) from that revealed for the control experiment. This finding suggests that the WSE of digestates did not affect cell viability or exert any cytotoxic effect on Caco-2 cells.

Tight junctions between intestinal epithelial cells are paramount in forming a regulated and specialized barrier between the intestinal lumen and the bloodstream (Sambuy et al., 2005). Indeed, food-derived peptides can cross the intestinal epithelium through tight junctions, and they can modify and modulate epithelium

permeability acting on protein profile of these junctions (Satake et al., 2002; Shimizu and Ok Son, 2007; Shimizu, 2010). For these reasons, we monitored TEER to check the integrity of the Caco-2 cell monolayers both before and after the transport experiments. No decline ($P > 0.05$) was found of TEER values during 1 h of incubation with PR digestates. At the end of transport experiment, the TEER values remained similar to those of the control experiment (400–500 Ωcm^2) and were comparable with data (>200 Ωcm^2) reported in other in vitro studies (Foltz et al., 2007).

To study the fate of ACE-I peptides present in the digested PR samples, we incubated Caco-2 monolayers cultivated onto a semi-permeable membrane of transwells with the 3-kDa WSE of digested PR samples. At the end of the transport experiment we analyzed the donor solution in the apical chamber and the receiving solution in the basolateral chamber by UPLC/ESI-HRMS. All the ACE-I peptides, which survived the in vitro GID of PR cheese, were susceptible to the action of Caco-2 peptidases, and 649.20 ± 148.85 mg/kg of LHLPLP remained in the apical compartment after 1 h of incubation (Supplemental Table S2; http://dx.doi.org/10.3168/jds.2015-9801). The total average amount of VPP (0.90 ± 0.18 mg/kg), IPP (1.10 ± 0.22 mg/kg), AYFYPEL (2.63 ± 0.43 mg/kg), AYFYPE (1.03 ± 0.40 mg/kg), and HLPLP (31.12 ± 3.50 mg/kg) was lower than 36.78 mg/kg. In the literature, the studied ACE-I peptides are reported to be differently sensitive to the action of cellular peptidases. For instance, the peptide LHLPLP is hydrolyzed in vitro by cellular peptidases to HLPLP (Quirós et al., 2008, 2009). On the contrary, this last peptide is not further degraded by brush border peptidases, and it remains intact even when incubated in vitro with human plasma (Quirós et al., 2008). The behavior of the synthetic peptides VPP and IPP has been studied by Ohsawa et al. (2008), who proved the 2 tripeptides to be fairly resistant to brush border peptidases expressed in vitro on Caco-2 cell surfaces.

At the end of the incubation, 0.6% (3.79 ± 1.95 mg/kg) of LHLPLP initially present in the 3-kDa WSE of digestates added to the apical compartment was transported intact to the basolateral chamber. In addition, the peptide HLPLP partially (2.9%, 0.89 ± 0.30 mg/kg) crossed the monolayer intact, as previously observed by Quirós et al. (2008). Accordingly, the apparent mean permeability coefficients (P_{app}) were 4.34×10^{-6} cm/s and 2.14×10^{-6} cm/s for LHLPLP and HLPLP, respectively. These values are in accordance with those reported for other food peptides, which ranged from 10^{-6} to 10^{-9} cm/s (Foltz et al., 2008; Contreras, et al., 2012). No other intact ACE-I peptides crossed the Caco-2 cell monolayer. However, we found several hydrolytic fragments of the studied ACE-I peptides in the basolateral compartment (data not shown). These findings somewhat disagree with those of Satake et al. (2002) who, using 1 m*M* solution of synthetic peptide, reported VPP to be transported intact across the Caco-2 cell monolayers via paracellular diffusion. Other in vitro experiments demonstrated the synthetic peptide AYFYPEL (1 m*M* solution) to be absorbed intact through Caco-2 cell monolayers (Contreras et al., 2012). Nonetheless, it is worth noting that our data refer to the transport of ACE-I peptides present in WSE of in vitro digestates of real cheese. For this reason, the crossing of LHLPLP and HLPLP revealed in this study better addresses further studies concerning the potential ACE-I activity of (digested) PR cheese.

CONCLUSIONS

This work reports for the first time the quantitative values of 8 different potent ACE-I peptides in PR cheese samples, in their gastrointestinal digestates and the transepithelial transport of these peptides present in digestates through the Caco-2 cell monolayer in vitro. The results support the formation and the degradation of some of the studied ACE-I peptides both during in vitro GID and transepithelial transport. Moreover, the obtained data demonstrate for the first time that certain ACE-I peptides present in WSE of PR digestates are partially transported intact across the Caco-2 monolayer in vitro. Overall, these findings provide important insights for assessing the potential bioactivity associated with the digestion of PR cheese.

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