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RESEARCH ARTICLE

The (193–209) 17-residues peptide of bovine β-casein is transported through Caco-2 monolayer

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Although the bioavailability of large peptides with biological activity is of great interest, the intestinal transport has been described for peptides up to only nine residues. β-casein (β-CN, 193–209) is a long and hydrophobic peptide composed of 17 amino acid residues (molecular mass 1881 Da) with immunomodulatory activity. The present work examined the transport of the β-CN (193–209) peptide across Caco-2 cell monolayer. In addition, we evaluated the possible routes of the β-CN (193–209) peptide transport, using selective inhibitors of the different routes for peptide transfer through the intestinal barrier. The results showed that the β-CN (193–209) peptide resisted the action of brush-border membrane peptidases, and that it was transported through the Caco-2 cell monolayer. The main route involved in transepithelial transport of the β-CN (193–209) peptide was transcytosis *via* internalized vesicles, although the paracellular transport *via* tight-junctions could not be excluded. Our results demonstrated the transport of an intact long-chain bioactive peptide in an *in vitro* model of intestinal epithelium, as an important step to prove the evidence for bioavailability of this peptide.

Keywords:
Caco2-cells / Immunomodulatory peptide / Intestinal transport / Mass spectrometry

1 Introduction

Milk proteins are a source of peptides that exhibit numerous bioactivities including antihypertensive, opiate, immunomodulatory, antimicrobial, antioxidant or mineral-binding activities [1–5]. Among those, the β-casein (β-CN, 193–209) peptide is released from the C-terminal end of β-CN by hydrolysis with pepsin. This peptide was isolated and identified from yoghurt and fermented milks as well as several types of cheese [6–8]. It is a 17 residues long peptide with the amino acid sequence Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val. This peptide displays immunomodulatory properties and shows mitogenic activity on primed lymph node cells and unprimed rat spleen cells [9]. It manifests chemotactic activity on L14 lymphoblastoid cell line [10], and enhances phagocytosis in rat macrophages [11, 12].

To exert their biological activity, some peptides have to cross the gastrointestinal barrier, and reach the circulation and target sites in an active form [13]. Resistance to enzymatic degradation and transport through intestinal cells are the two important factors influencing the bioavailability of orally ingested peptides. There are some distinctive features determining the possibility of a peptide to be absorbed intact through the intestinal epithelium, such as its molecular mass, hydrophobicity, charge or tendency to aggregate [14, 15]. Interestingly, the presence of four proline residues within the sequence can protect the long

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Abbreviations: β-CN, β-casein; **TEER,** transepithelial electrical resistance; **TM,** transport medium; **UV,** ultraviolet

β-CN (193–209) peptide from the action of peptidases. As a consequence, this peptide appears as a good candidate for crossing the intestinal barrier in an intact bioactive form. The main routes recognized for transepithelial absorption of peptide in the gut include the PepT1 transporter-mediated transport for di- and tri-peptides, the paracellular passive transport *via* tight junctions, the transcellular passive diffusion and transcytosis that is a transcellular route involving endocytotic uptake, intracellular transport *via* transcytotic vesicles and basolateral secretion [16].

The aim of this study was to determine the sensitivity of the β-CN (193–209) peptide to hydrolysis by brush border enzymes and its transepithelial transport across Caco-2 cell monolayer as a model of intestinal epithelium. The pathway of transepithelial transport was investigated by using selective inhibitors of the different routes, including the dipeptide Gly-Pro that competitively inhibits the peptide transporter PepT1 [14], cytochalasin D that opens tight junctions by altering the cytoskeletal structure [17] and increasing the passive paracellular route, and wortmannin as an inhibitor of transcytosis [18].

2 Materials and methods

2.1 Chemicals

DMEM, non-essential amino acids, gentamycin sulfate, Hank's balanced salt solution and PBS were purchased from Lonza, Switzerland. l-Glutamine and trypsine-EDTA were purchased from Gibco (Invitrogen, France). Fetal calf serum was supplied by Dutscher, France. Cytochalasin D, Glycil-Proline dipeptide (Gly-Pro), neutral red powder, glycine, glucose, HEPES, DMSO and trinitrobenzenesulfonic acid were supplied by Sigma–Aldrich, France. Wortmannin was obtained from LC Laboratories, MA, USA. Acetonitrile and TFA were purchased from Fluka, France.

2.2 Preparation of the β-CN (193–209) peptide

The peptide was obtained in a purified form as previously described [19]. Conditions for hydrolyzing β-CN were slightly modified for β-CN concentration (5 g/L), molar ratio chymosin/β-CN (1/8000) and duration of hydrolysis (150 min). Then, the reaction was stopped by heat inactivation of the enzyme (80°C, 15 min). The pH of the mixture was subsequently adjusted to 4.6 with 1 M HCl to precipitate and remove by centrifugation ($7000 \times g$ for 20 min) whole casein and its large fragments. After readjusting the pH to 6.5, the supernatant was ultra-filtered (Spiral-wound UF cartridge S10T3 MWCO 3 Kda; Amicon, Lexington, MA, USA) and the ultra-filtrate was concentrated with a membrane (Filtron membrane 1 KDa) and freeze-dried. The β-CN (193–209) peptide, identified by ESI/MS, was obtained with a purity of 98% estimated by RP-HPLC-ESI/MS as described in Section 2.5.

2.3 Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM supplemented with 20% fetal calf serum, 1% non-essential amino acids, 2 mM L-Glutamine and 25 μg/mL gentamycin sulphate. They were incubated at 37°C in humidified atmosphere containing 5% CO_2. The monolayer became confluent 4–5 days after seeding 3 000 000 cells/flask (75 cm^2 flasks, Greiner Bio-one, France), and the cells were subcultured at split ratio of 1:5 by trypsinization (0.5% trypsin and 0.05% EDTA). The medium was changed every second day. The cells used in this study were at passage 35–45. For transport studies, cells were seeded in cell culture inserts with Anopore® membranes (0.2 μm pore sizes; 25 mm diameter; 4.7 cm^2 grown surface, from Nunc, Denmark) at 450 000 cells $\times$ cm^2 density and incubated in six-well culture plates. The medium was changed every 2 days. The monolayer became confluent after 4 days, and the cells differentiated for another 21 days before performing transepithelial transport experiments. The integrity of the cell layer was evaluated by transepithelial electrical resistance (TEER) measurement with EVOM epithelial volt-ohm meter (World Precision Instruments, FL, USA). Only Caco-2 monolayers showing TEER higher than $300\Omega \times cm^2$ were used for the experiments.

The integrity of the monolayers was checked before, during and after the experiment. TEER values remained stable around $300\,\Omega \times cm^2$ and no reduction was observed following the incubation with the peptide. Neither the β-CN (193–209) peptide administration nor incubation with inhibitors affected cellular viability that, at the end of the experiments, was not significantly different from the viability of the control (cell monolayers without β-CN (193–209) peptide, assessed at the beginning of the experiments).

2.4 Transepithelial transport studies

After TEER measurement, Caco-2 cells monolayers were gently rinsed twice with PBS, and transport medium (TM, Hank's balanced salt solution supplemented with 25 mM glucose and 10 mM HEPES) was added to the apical (2 ml) and to the basolateral (2 mL) compartments. After 30 min of incubation, medium was replaced with fresh TM containing 0, 0.5, 1, 2 or 4 mM of β-CN (193–209) peptide. The inserts were incubated at 37°C for 120 min and the apical and basolateral solutions were sampled at the beginning and at the end of incubation period for RP-HPLC-ESI/MS analyses to measure the concentration of the β-CN (193–209) peptide in both compartments. For inhibition experiments, Gly-Pro (5, 10, 20 mM) was dissolved in TM,

and wortmannin (0.25, 0.5, 1 μM) and cytochalasin D (0.25, 0.5, 1 μg/mL) were dissolved in DMSO and immediately diluted in TM (0.05% DMSO final concentration). The cell monolayers were incubated for 30 min with the inhibitors or with 0.05% DMSO, as a control, before the peptide transport experiments. During and at the end of the experiments, TEER was measured and data were recorded only from experiments in which TEER was higher than 250 Ω/cm^2. To exclude that addition of the β-CN (193–209) peptide and/or inhibitors could be toxic for the cells, cellular viability was assessed at the end of each experiment using the vital dye neutral red, according to the manufacturer's instructions.

2.5 RP-HPLC-ESI/MS analyses

Analytical RP-HPLC was carried out using Agilent HP1100 chromatographic system (Agilent Technologies, Massy, France). Separations were performed on a narrow-bore Symmetry C_{18} column (5 μm particle size, 2.1 × 150 mm, Waters, WAT 056975, Milford, MA, USA), equipped with a C_{18} cartridge guard. The elution was run at 0.25 mL/min and 40°C by a binary gradient with acetonitrile as an organic modifier. Solvent A contained 0.106% TFA in Milli Q water (v/v) and solvent B contained 0.1% TFA in acetonitrile-Milli Q water (80:20, v/v). Samples were analyzed by on-line RP-HPLC-ESI/MS. The column was initially equilibrated with 10% of solvent B. Samples were applied to the column and eluted by a linear gradient of solvent B performed as follows: 0–25 min, 10–70%; 25–27 min 100%, the column was held at 100% during 3 min and then equilibrated at 10% during 10 min. Throughout on-line coupling, splitting of chromatographic flow was achieved by a low dead volume with 85% of the flow directed to the ultraviolet (UV) detector and 15% to the mass spectrometer. This split allows a perfect superposition between UV and TIC detection. Peaks were detected both by UV absorbance at 214 nm and peptide MS by TIC.

The β-CN (193–209) peptide was quantified in accordance to a standard curve established with chosen quantities (from 0.053 to 1.063 nmol) of purified β-CN (193–209) peptide. β-CN (193–209) quantity (x) in apical and basolateral solutions was calculated with the equation $y = bx + c$, where y is the UV absorbance at 214 nm, c is the y-axis intercept and b the slope of the standard curve.

The proteolysis of the peptide was analyzed by LC-MS. The mass spectrometer (API III+SCIEX, Thornhill, ON, Canada) comprises a triple quadrupole equipped with an atmospheric pressure ionization source. Analysis was carried out in positive detection mode. A 75 μm sprayer was usually set at 4800 V for generated multiply-charged ions and orifice set between 60 and 90 V depending on experiments. The nebulizer pressure was set around 0.5 MPa and the curtain gas set to 1.2 L/min. The instrument mass-to-charge (m/z) scale was calibrated with polypropylene glycols. All peptide mass spectra were obtained from the average signal of multiple scans. Each scan was acquired over the range of m/z values from 500 to 2000 using a step size of 0.5 Da and a dwell time of 0.5 ms. The measured masses were matched with predicted enzymatic fragments by using the software BioMultiview 1.3.1 (MDS Perkin Elmer Sciex, Thornhill, Canada).

2.6 Data analysis

The results were expressed as the mean values of at least three independent experiments. The basolateral concentration of the β-CN (193–209) peptide in function of its administered apical concentration was subjected to regression analysis using the Logistic Dose-Response interpolation equation (four parameters) provided by Table-Curve2D software program (Jandel Scientific, San Rafael, CA, USA). The effect of the inhibitors on the β-CN (193–209) peptide flux was evaluated by analysis of variance. The differences between each experimental condition and the control were analyzed by the Dunnett test (Statgraphics Plus 4; Manugistics, MD, USA). Differences with p-values < 0.05 were considered as significant.

3 Results

3.1 Transepithelial transport of the β-CN (193–209) peptide across a Caco-2 monolayer

The RP-HPLC-ESI/MS analysis and the standard curve generated using pure β-CN (193–209) peptide permitted the quantification of this peptide in apical and basolateral solutions (Fig. 1). We verified using LC-MS/MS that the β-CN (193–209) peptide was the sole one present in the apical solution at the beginning of the incubation. After 120 min incubation, the peptide was not significantly hydrolyzed by the brush border exopeptidases, and the products of hydrolysis were the β-CN (194–209) and β-CN (193–208) peptides (Fig. 2). The hydrolysis of the β-CN (193–209) peptide in apical solution was quantitatively limited over the experimental duration and regardless of the peptide concentration (Figs. 3A and B). When the peptide was added at 2 mM, a 10% decrease in its concentration in the apical compartment was observed after 2 h incubation, and the peptide left remained intact. For higher concentrations, the hydrolysis was less than 10%.

RP-HPLC-ESI/MS analysis of basolateral solution showed that the β-CN (193–209) peptide and its two derived fragments were absorbed intact through Caco-2 monolayer. After 120 min incubation in the apical compartment at the milli molar range, the β-CN (193–209) peptide appeared in basolateral compartment at the micro-molar range with concentration values following a saturable pattern (Fig. 4), described by a sigmoidal curve.

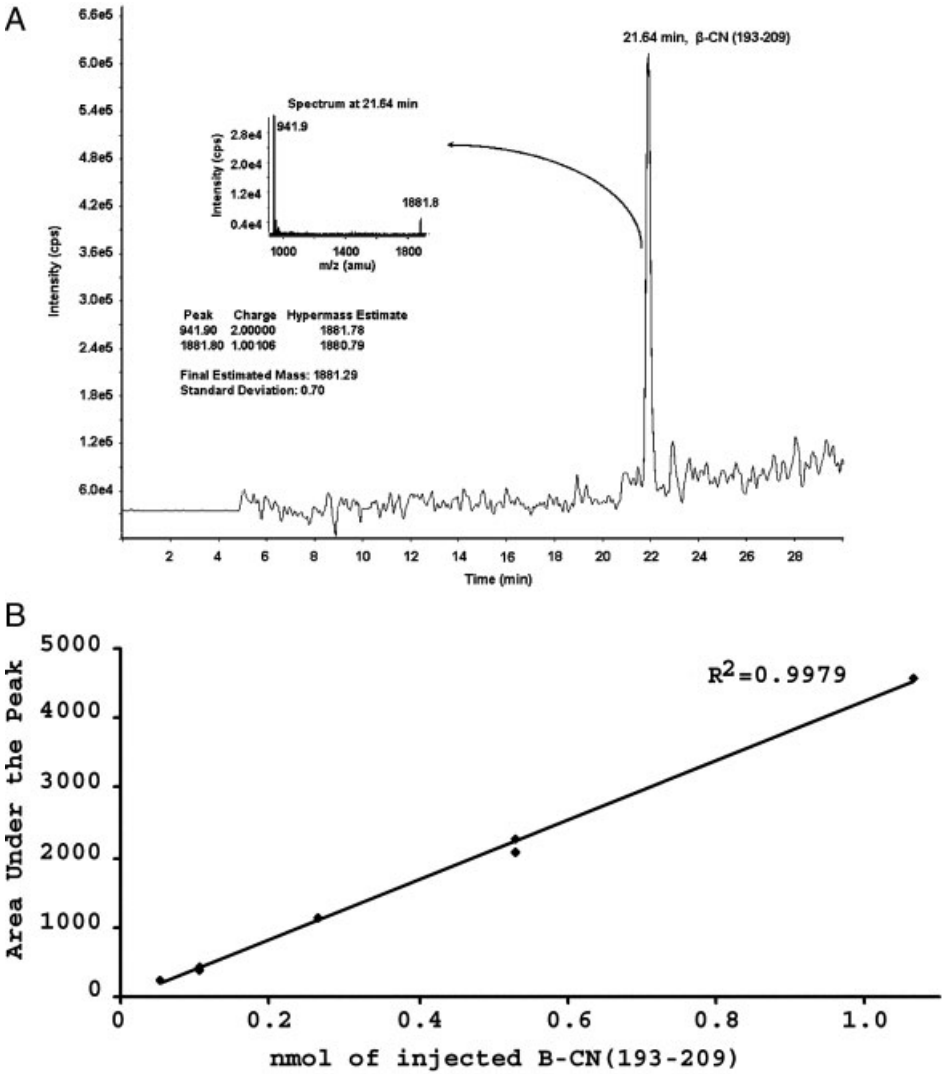

Figure 1. Identification and quantification of the β-CN (193–209) peptide by RP-HPLC-ESI/MS analysis. (A) Identification and estimation of β-CN (193–209) purity level are shown on the spectrum and on the TIC graph. The β-CN (193–209) peptide was added in the apical compartment at 2 mM and apical solution immediately analyzed. (B) Quantification of the peptide in apical and basolateral solutions using a five-point calibration curve of pure β-CN (193–209) peptide as standard analyzed by RP-HPLC-ESI/MS (see Section 2.5).

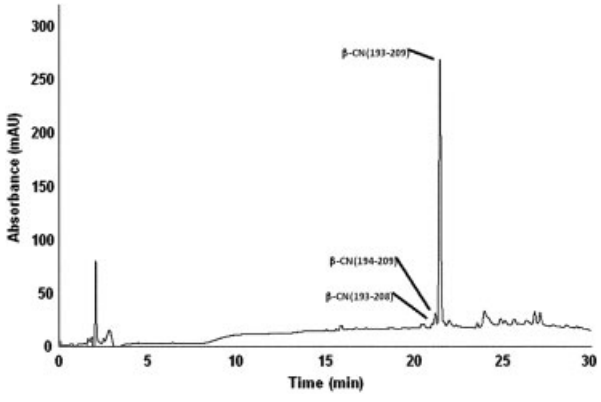

Figure 2. RP-HPLC-ESI/MS analysis of the apical solution after 120 min of incubation. The β-CN (193–209) peptide was previously added (2 mM) in the apical compartment at time 0. During the incubation with Caco-2 monolayer, β-CN (194–209) and β-CN (193–208) peptides were generated from β-CN (193–209) peptide hydrolysis.

3.2 Influence of Gly-Pro, Cytochalasin D and wortmannin on β-CN (193–209) peptide transport

To evaluate the pathway of the transepithelial transport of the β-CN (193–209) peptide, the effect of some inhibitors on the apical to basolateral flux of the peptide was tested (Fig. 5). The transport of the β-CN (193–209) peptide was not significantly decreased by Gly-Pro (applied from 5 to 20 mM) that competitively inhibits the peptide transporter PepT1. In the range 0.25 to 1 μg/mL the treatment with cytochalasin D, a tight-junctions disruptor, reduced TEER values by approximately 20% compared to the control, indicating that paracellular route was similarly expanded regardless of the concentration of chytochalasin D. Nevertheless, the presence of cytochalasin D at 0.25, 0.5 and 1 μg/mL did not significantly alter apical to basolateral flux of the β-CN (193–209) peptide. On the contrary, the addition of the inhibitor of transcytosis wortmannin in the range 0.25–1 μM significantly ($p < 0.05$) reduced

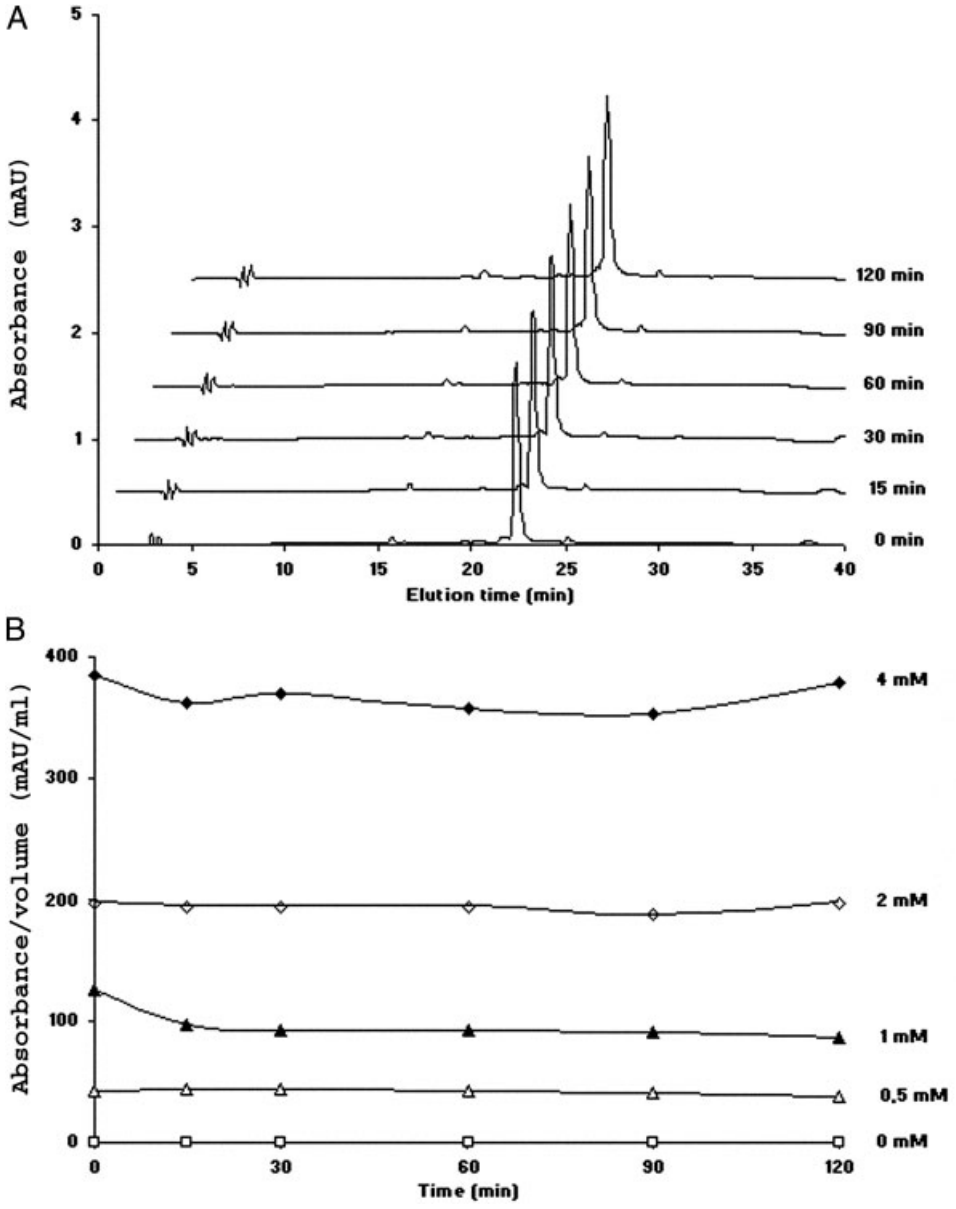

Figure 3. Stability of the β-CN (193–209) peptide at the apical compartment of Caco-2 cell monolayer. (A) LC-chromatograms obtained from RP-HPLC-ESI/MS analysis of the apical solution in the presence of 2 mM of the β-CN (193–209) peptide from 0 to 120 min. The peak eluted at 22 min corresponds to the peptide. (B) Change in peak height of the β-CN (193–209) peptide introduced at different concentrations in the apical compartment of Caco-2 cell monolayer, as determined from LC chromatograms.

the flux of the peptide through the Caco-2 monolayer and an average 53% decrease of transport was determined (Fig. 5).

4 Discussion

The hypothesis that peptides escape digestion and are transported from the intestinal lumen into blood circulation is gaining acceptance for small peptides, mainly due to the growing number of studies describing the *in vitro* transepithelial transport of bioactive peptides [13, 20–29]. In the present study we demonstrate that the 17 residues β-CN (193–209) peptide and the derived β-CN (193–208) and β-CN (194–209) peptides are transported across Caco-2 cell monolayer, with the major contribution of the transcytosis mechanisms.

To exert its biological effects an ingested peptide must first resist intestinal hydrolysis. To study the resistance of the β-CN (193–209) peptide to brush-border membrane peptidases, we used Caco-2 cell monolayer because, under specific culture conditions, Caco-2 cells undergo a process of differentiation leading to the expression of several morphological and functional characteristics of the enterocyte including the microvillus structure and of brush-border enzymes in the apical membrane [30, 31]. About 10% of the β-CN (193–209) peptide were hydrolyzed through the action of amino- and carboxypeptidases present on the apical membrane, but the β-CN (193–209) peptide and the two derived peptides, β-CN (193–208) and β-CN (194–209), were not further hydrolyzed by brush-border membrane exopeptidases. Moreover, the three peptides resisted the

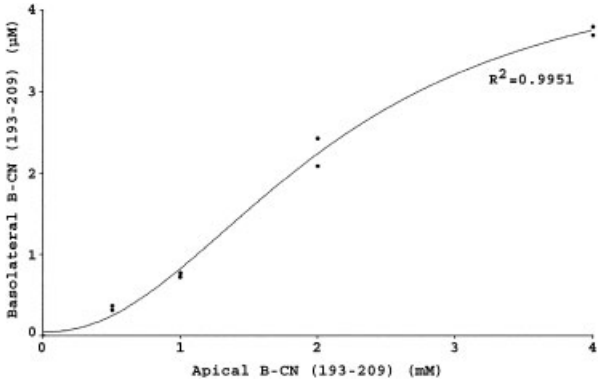

Figure 4. Concentration-dependent transport of the β-CN (193–209) peptide across Caco-2 monolayer. Different amounts of peptide were incubated in apical compartment and β-CN (193–209) evaluated in basolateral solution after 120 min incubation at 37°C. Quantification was as described in Section 2.5.

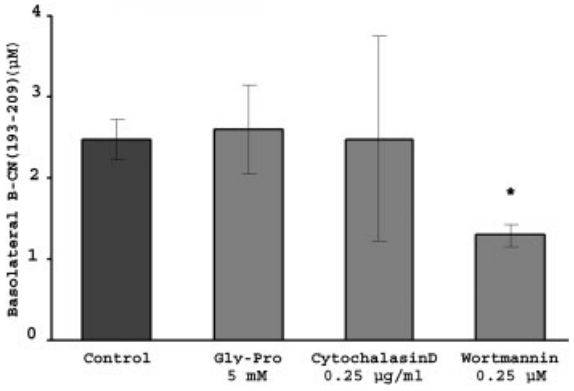

Figure 5. Effects of Gly-Pro (a competitive inhibitor of peptide transporter PepT1), cytochalasin D (a disrupter of tight junction) and wortmannin (an inhibitor of transcytosis) on transport of 2 mM of β-CN (193–209) peptide across the Caco-2 cell monolayers. Results are expressed as the mean ± SEM ($n = 3$). Means were compared with the control using the Dunnett test ($^{*}p < 0.05$).

action of intracellular peptidases. In general, due to their rapid hydrolysis by the brush border or cytoplasmic peptidases, the bioavailability of two to nine residues-peptides is extremely low [13, 21, 23, 24, 26, 28, 29, 32]. The resistance of the β-CN (193–209) peptide to the action of Caco-2 brush-border peptidases is possibly related to its proline-rich sequence (four proline residues on 17 residues), and other proline-containing peptides were found to be resistant to intestinal proteolysis [33, 34]. This finding is further confirmed by Savoie *et al.* [35], who observed that peptides rich in proline and glutamic acid are more resistant to pepsin and pancreatin activity, suggesting that the β-CN (193–209) peptide would resist gastric and duodenal digestion. This hypothesis was affirmed by a regular appearance of the β-CN (193–209) peptide among the main peptides released from the stomach of milk-fed calf [36]. Thereafter this peptide appears in the intestinal lumen where it can be absorbed. To our knowledge no data exist on the hydrolysis of the C-terminal end of β-CN in human fed a milk diet.

Caco-2 cells cultured on a semi permeable filter were used to demonstrate that the β-CN (193–209) peptide could be transported trough the intestinal barrier. Moreover, additional experiments using selective inhibitors of the different routes for the transepithelial transport of the β-CN (193–209) peptide suggested the involvement of transcytosis among the different transport pathways. Caco-2 cells have been used for the present study because they express the carrier-mediated transport systems for amino acids and di- and tri-peptides [37, 38], show a transcytotic activity [39] and develop tight junctions that are involved in the paracellular route [31, 40]. Our results did not permit to exclude a possible involvement of the paracellular route in the transport of the β-CN (193–209) peptide because of large standard deviation observed when cytochalasin D was administrated to the cells. In addition, the 20% reduction of the TEER might be insufficient to increase the paracellular transport of macromolecules. Nevertheless the fact that the β-CN (193–209) peptide was mainly transported by transcytosis is possibly related to its physico-chemical characteristic: it is a large and hydrophobic peptide, negatively charged under the experimental conditions (neutral pH). So the passive paracellular transport *via* tight junctions was not the probable route because it is normally applicable to the absorption of water-soluble low-molecular-weight substances [41] and short-chain peptides [21, 23, 42, 43] and, in general, it is specific for positively charged molecules because tight junctions are overall negatively charged [44]. Regarding the transcellular route, our results showed that the transporter PepT1 was not involved in the transport of the β-CN (193–209) peptide across Caco-2 cell monolayer. This result reinforces the assumption that this large peptide have only little possibility to be transported by the H+-coupled PepT1 transporter because PepT1 is an active and saturable symporter known for intestinal absorption of di- and tri-peptides [14, 15, 45]. The low level of degradation of the peptide during its transepithelial transfer strongly suggests that passive transcellular diffusion is not the main pathway involved in the transport of the peptide. In contrast, the significant reduction of the transport in the presence of wortmannin indicated transcytosis as a potential candidate for the transepithelial transport of the β-CN (193–209) peptide [18].

Simultaneously to its identification, the β-CN (193–209) peptide was quantified in apical and basolateral compartments using RP-HPLC-ESI/MS analysis. The concentration of peptide absorbed was 0.2–0.3 mM, even if higher concentrations were applied in the apical compartment. From these results obtained *via* a model approach, it is difficult to evaluate whether the absorption of the peptide when present in food matrix would be comparable. Nevertheless, the food matrix such as cheese will be extensively disorganized when reaching the small intestine. After 120 min incubation, the β-CN (193–209) peptide appeared in the basolateral compartment at 2 µM. Thus the actual amount of peptide transepithelially transported was about

1%. This value is similar to the one determined for the antihypertensive tripeptide Val-Pro-Pro of which 2% was transported from the apical to the basolateral side [21]. In contrast to our 17-residues peptide, 87% of the tripeptide were absorbed by the cells. Consequently the absorption *via* endocytosis appears as to be the limiting step in the transepithelial transport of the long peptide. The low amount of β-CN (193–209) peptide absorbed is probably linked to its physico-chemical characteristics, in particular its hydrophobicity [46, 47]. Assuming that the β-CN (193–209) peptide is transported mainly through a transcytosis route, a vesicular-mediated internalization, the mechanism involved is probably absorption by apical cell membrane through hydrophobic interactions [48]. Moreover, considering the presence of arginine residue in its sequence, the β-CN (193–209) peptide could form hydrogen bonds with lipid phosphates of cell membranes, thus favoring the translocation process *via* transcytosis route [49]. A similar mechanism has been described for the absorption of some peptides, as bradykinin that is a nine residues peptide with three proline residues and basic oligopeptides [23, 32, 50].

In conclusion, this study demonstrated the transepithelial transport of the β-CN (193–209) peptide, a long and hydrophobic peptide across a well-known *in vitro* model of intestinal epithelium. The significant inhibitory effect of wortmannin on the transepithelial transport of β-CN (193–209) peptide suggests that transcytosis is the most important mechanism of transport for the peptide through the Caco-2 cells monolayer, even if other mechanism of transport cannot be completely excluded. It remains to confirm the exact molecular mechanism underlying β-CN (193–209) peptide translocation into the cells to more precisely identify the tissue target for this peptide to exert a regulatory physiological effect. As a consequence, to visualize the translocation steps would be of crucial importance to characterize the route for intestinal β-CN (193–209) passage.

The authors have declared no conflict of interest.

5 References

[1] Nakamura, Y., Yamamoto, N., Sakai, K., Takano, T., Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* 1995, *78*, 1253–1257.

[2] Meisel, H., Biochemical properties of peptides encrypted in bovine milk proteins. *Curr. Med. Chem.* 2005, *12*, 1905–1919.

[3] Laffineur, E., Genetet, N., Leonil, J., Immunomodulatory activity of beta-casein permeate medium fermented by lactic acid bacteria. *J. Dairy Sci.* 1996, *79*, 2112–2120.

[4] Vermeirssen, V., Augustijns, P., Morel, N., Van Camp, J. *et al.*, *In vitro* intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests. *Int. J. Food Sci. Nutr.* 2005, *56*, 415–430.

[5] Rutherfurd-Markwick, K. J., Moughan, P. J., Bioactive peptides derived from food. *J. AOAC Int.* 2005, *88*, 955–966.

[6] Dionysius, D. A., Marschke, R. J., Wood, A. J., Milne, J. *et al.*, Identification of physiologically functional peptides in dairy products. *Aust. J. Dairy Tech.* 2000, *55*, 103.

[7] Hernandez-Ledesma, B., Amigo, L., Ramos, M., Recio, I., Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *J. Agric. Food Chem.* 2004, *52*, 1504–1510.

[8] Gagnaire, V., Molle, D., Herrouin, M., Leonil, J., Peptides identified during Emmental cheese ripening: origin and proteolytic systems involved. *J. Agric. Food Chem.* 2001, *49*, 4402–4413.

[9] Coste, M., Rochet, V., Leonil, J., Molle, D. *et al.*, Identification of *C*-terminal peptides of bovine beta-casein that enhance proliferation of rat lymphocytes. *Immunol. Lett.* 1992, *33*, 41–46.

[10] Frontenau, D., Tanneau, G., Henry, G., Chevaleyre, *et al.*, Activité chimiotactique de lait d'artiodactyle sur les lymphocytes porcins. *Journées Rech. Porcine en France* 1998, *30*, 363–367.

[11] Sandre, C., Gleizes, A., Forestier, F., Gorges-Kergot, R. *et al.*, A peptide derived from bovine beta-casein modulates functional properties of bone marrow-derived macrophages from germfree and human flora-associated mice. *J. Nutr.* 2001, *131*, 2936–2942.

[12] Unanue, E. R., Allen, P. M., The basis for the immunoregulatory role of macrophages and other accessory cells. *Science* 1987, *236*, 551–557.

[13] Vermeirssen, V., Deplancke, B., Tappenden, K. A., Van Camp, J. *et al.*, Intestinal transport of the lactokinin Ala-Leu-Pro-Met-His-Ile-Arg through a Caco-2 Bbe monolayer. *J. Pept. Sci.* 2002, *8*, 95–100.

[14] Fei, Y. J., Kanai, Y., Nussberger, S., Ganapathy, V. *et al.*, Expression cloning of a mammalian proton-coupled oligopeptide transporter. *Nature* 1994, *368*, 563–566.

[15] Aito-Inoue, M., Lackeyram, D., Fan, M. Z., Sato, K. *et al.*, Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane. *J. Pept. Sci.* 2007, *13*, 468–474.

[16] Shimizu, M., Modulation of intestinal functions by food substances. *Nahrung/Food* 1999, *43*, 154–158.

[17] Madara, J. L., Barenberg, D., Carlson, S., Effects of cytochalasin D on occluding junctions of intestinal absorptive cells: further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. *J. Cell Biol.* 1986, *102*, 2125–2136.

[18] Hansen, S. H., Olsson, A., Casanova, J. E., Wortmannin, an inhibitor of phosphoinositide 3-kinase, inhibits transcytosis in polarized epithelial-cells. *J. Biol. Chem.* 1995, *270*, 28425–28432.

[19] Bouhallab, S., Molle, D., Leonil, J., Continuous hydrolysis of beta-casein in a membrane reactor - Preparation of a bioactive peptide. *Biotechnol. Lett.* 1993, *15*, 697–702.

[20] Zhu, X. L., Watanabe, K., Shiraishi, K., Ueki, T. *et al.*, Identification of ACE-inhibitory peptides in salt-free soy sauce

that are transportable across caco-2 cell monolayers. *Peptides* 2008, *29*, 338–344.

[21] Satake, M., Enjoh, M., Nakamura, Y., Takano, T. *et al.*, Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers. *Biosci. Biotechnol. Biochem.* 2002, *66*, 378–384.

[22] Sienkiewicz-Szlapka, E., Jarmolowska, B., Krawczuk, S., Kostyra, E. *et al.*, Transport of bovine milk-derived opioid peptides across a Caco-2 monolayer. *Int. Dairy J.* 2009, *19*, 252–257.

[23] Shimizu, M., Tsunogai, M., Arai, S., Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2. *Peptides* 1997, *18*, 681–687.

[24] Quiros, A., Davalos, A., Lasuncion, M. A., Ramos, M. *et al.*, Bioavailability of the antihypertensive peptide LHLPLP: Transepithelial flux of HLPLP. *Int. Dairy J.* 2008, *18*, 279–286.

[25] Miguel, M., Davalos, A., Manso, M. A., de la Pena, G. *et al.*, Transepithelial transport across Caco-2 cell monolayers of antihypertensive egg-derived peptides. PepT1-mediated flux of Tyr-Pro-Ile. *Mol. Nutr. Food Res.* 2008, *52*, 1507–1513.

[26] Lei, L., Sun, H. Y., Liu, D., Liu, L. G. *et al.*, Transport of Val-Leu-Pro-Val-Pro in human intestinal epithelial (Caco-2) cell monolayers. *J. Agric. Food Chem.* 2008, *56*, 3582–3586.

[27] Iwan, M., Jarmolowska, B., Bielikowicz, K., Kostyra, E. *et al.*, Transport of mu-opioid receptor agonists and antagonist peptides across Caco-2 monolayer. *Peptides* 2008, *29*, 1042–1047.

[28] Foltz, M., Cerstiaens, A., van Meensel, A., Mols, R. *et al.*, The angiotensin converting enzyme inhibitory tripeptides Ile-Pro-Pro and Val-Pro-Pro show increasing permeabilities with increasing physiological relevance of absorption models. *Peptides* 2008, *29*, 1312–1320.

[29] Bernasconi, E., Fritsche, R., Corthesy, B., Specific effects of denaturation, hydrolysis and exposure to *Lactococcus lactis* on bovine beta-lactoglobulin transepithelial transport, antigenicity and allergenicity. *Clin. Exp. Allergy* 2006, *36*, 803–814.

[30] Howell, S., Kenny, A. J., Turner, A. J., A survey of membrane peptidases in 2 human colonic cell-lines, Caco-2 and Ht-29. *Biochem. J.* 1992, *284*, 595–601.

[31] Hidalgo, I. J., Raub, T. J., Borchardt, R. T., Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989, *96*, 736–749.

[32] Sai, Y., Kajita, M., Tamai, I., Kamata, M. *et al.*, Intestinal absorption of fluorescence-derivatized cationic peptide 001-C8-NBD via adsorptive-mediated transcytosis. *Bioorg. Med. Chem.* 1998, *6*, 841–848.

[33] Cardillo, G., Gentilucci, L., Tolomelli, A., Calienni, M. *et al.*, Stability against enzymatic hydrolysis of endomorphin-1 analogues containing beta-proline. *Org. Biomol. Chem.* 2003, *1*, 1498–1502.

[34] Mizuno, S., Nishimura, S., Matsuura, K., Gotou, T. *et al.*, Release of short and proline-rich antihypertensive peptides from casein hydrolysate with an *Aspergillus oryzae* protease. *J. Dairy Sci.* 2004, *87*, 3183–3188.

[35] Savoie, L., Agudelo, R. A., Gauthier, S. F., Marin, J. *et al.*, *In vitro* determination of the release kinetics of peptides and free amino acids during the digestion of food proteins. *J. AOAC Int.* 2005, *88*, 935–948.

[36] Yvon, M., Pelissier, J. P., Characterization and kinetics of evacuation of peptides resulting from casein hydrolysis in the stomach of the calf. *J. Agric. Food Chem.* 1987, *35*, 148–156.

[37] Thwaites, D. T., Brown, C. D., Hirst, B. H., Simmons, N. L., H(+)-coupled dipeptide (glycylsarcosine) transport across apical and basal borders of human intestinal Caco-2 cell monolayers display distinctive characteristics. *Biochim. Biophys. Acta* 1993, *1151*, 237–245.

[38] Saito, H., Inui, K., Dipeptide transporters in apical and basolateral membranes of the human intestinal-cell line Caco-2. *Am. J. Physiol.* 1993, *265*, G289–G294.

[39] Heyman, M., Crain-Denoyelle, A. M., Nath, S. K., Desjeux, J. F., Quantification of protein transcytosis in the human colon carcinoma cell line CaCo-2. *J. Cell. Physiol.* 1990, *143*, 391–395.

[40] Hashimoto, K., Shimizu, M., Epithelial properties of human intestinal Caco-2 cells cultured in a serum-free medium. *Cytotechnology* 1993, *13*, 175–184.

[41] Karbach, U., Paracellular calcium-transport across the small-intestine. *J. Nutr.* 1992, *122*, 672–677.

[42] Pappenheimer, J. R., Dahl, C. E., Karnovsky, M. L., Maggio, J. E., Intestinal-absorption and excretion of octapeptides composed of D-amino-acids. *Proc. Natl. Acad. Sci. USA* 1994, *91*, 1942–1945.

[43] Adson, A., Raub, T. J., Burton, P. S., Barsuhn, C. L. *et al.*, Quantitative approaches to delineate paracellular diffusion in cultured epithelial-cell monolayers. *J. Pharm. Sci.* 1994, *83*, 1529–1536.

[44] Salamat-Miller, N., Johnston, T. P., Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium. *Int. J. Pharm.* 2005, *294*, 201–216.

[45] Brandsch, M., Knutter, I., Bosse-Doenecke, E., Pharmaceutical and pharmacological importance of peptide transporters. *J. Pharm. Pharmacol.* 2008, *60*, 543–585.

[46] Pauletti, G. M., Okumu, F. W., Borchardt, R. T., Effect of size and charge on the passive diffusion of peptides across Caco-2 cell monolayers *via* the paracellular pathway. *Pharm. Res.* 1997, *14*, 164–168.

[47] Pauletti, G. M., Gangwar, S., Knipp, G. T., Nerurkar, M. M. *et al.*, Structural requirements for intestinal absorption of peptide drugs. *J. Control. Release* 1996, *41*, 3–17.

[48] Knipp, G. T., Velde, D. G. V., Siahaan, T. J., Borchardt, R. T., The effect of beta-turn structure on the passive diffusion of peptides across Caco-2 cell monolayers. *Pharm. Res.* 1997, *14*, 1332–1340.

[49] Zhao, K., Luo, G., Zhao, G. M., Schiller, P. W. *et al.*, Transcellular transport of a highly polar 3+ net charge opioid tetrapeptide. *J. Pharmacol. Exp. Ther.* 2003, *304*, 425–432.

[50] Sai, Y., Kajita, M., Tamai, I., Wakama, J. *et al.*, Adsorptive-mediated endocytosis of a basic peptide in enterocyte-like Caco-2 cells. *Am. J. Physiol.* 1998, *275*, G514–G520.

