Food Chemistry 139 (2013) 203–212

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Transport across Caco-2 monolayers of peptides arising from *in vitro* digestion of bovine milk proteins

Gianluca Picariello [a,b,*], Giuseppe Iacomino [a], Gianfranco Mamone [a], Pasquale Ferranti [a,b], Olga Fierro [a], Carmen Gianfrani [a,c], Aldo Di Luccia [a,d], Francesco Addeo [a,b]

[a] Istituto di Scienze dell'Alimentazione (ISA), CNR, Via Roma 64, 83100 Avellino, Italy
[b] Dipartimento di Scienza degli Alimenti, Università di Napoli "Federico II", Parco Gussone, Portici (NA) 80055, Italy
[c] European Laboratory for the Investigation of Food Induced Diseases (ELFID), Università di Napoli "Federico II", Napoli, Italy
[d] Dipartimento di Scienze Agrarie, degli Alimenti e dell'Ambiente, Università di Foggia, Via Napoli 25, 71100 Foggia, Italy

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form 5 January 2013
Accepted 8 January 2013
Available online 4 February 2013

Keywords:
Milk proteins
Gastrointestinal digestion
Caco-2 cell monolayers
Bioactive peptides
Cow's milk allergy
Peptide uptake

ABSTRACT

The entire panel of peptides produced from caseins (CN) and whey proteins (WP) that survive *in vitro* sequential gastro-pancreatic digestion and translocate across monolayers of Caco-2 cells, used as a model of the intestinal epithelium, has been characterised by HPLC and mass spectrometry. Among the milk-derived bioactive peptides, only minor amounts of mono-phosphorylated peptides arising from α_s1- and β-CN were detected. The absorption behaviour of two resistant β-lactoglobulin (β-Lg) domains, β-Lg 125–135 and β-Lg 40–60, was studied in detail using synthetic peptides. The IgE-binding properties of the digests recovered from the apical and basolateral monolayer compartments were evaluated by dot-blot, using the sera of milk allergic children ($N = 5$). Outcomes indicated β-Lg 127–135 as a possible "immune sensitising factor" *in vivo*. The almost complete loss of the IgE-affinity of CN and WP after digestion points out the need to design *in vivo* experiments to track the metabolic fate of dietary proteins.

1. Introduction

Many studies have demonstrated that peptide sequences encrypted in food proteins can significantly influence a range of physiological functions (Clare & Swaisgood, 2000; Korhonen & Philanto, 2006; Meisel, 2005; Silva & Xavier Malcata, 2005). Food-derived bioactive peptides are effective as long as they are released by proteolytic events or during gastrointestinal (GI) digestion *in vivo*. The ability of bioactive peptides to reach their target site(s) unhydrolysed is a prerequisite for exerting any physiological effects. Therefore, it is clear that the proteolytic pathways of GI digestion have a decisive impact on the formation and/or degradation of specific bioactive sequences. The actual physiological involvement of food-derived peptides is uncertain in most cases since almost all biological tests have assayed sequences released *in vitro* under conditions that are very different from those occurring *in vivo*.

Milk proteins are by far the most extensively investigated source of food-derived bioactive peptides, although active sequences have been described from several food matrices. Several experimental approaches have attempted to reproduce more or less closely the conditions of the GI digestion of milk proteins to trace the pathways of their breakdown. However, none of these methods has yet been generally accepted and standardised (Hur, Lim, Decker, & McClements, 2011). The pattern of resistant peptides is expectedly affected by a certain degree of variability, depending on the conditions of the GI digestion model (Defernez, Mandalari, & Mills, 2010; Mandalari et al., 2009). Nevertheless, an overview of the recent literature confirms that some milk protein domains exhibit an intrinsic relative stability to hydrolysis. The domains of caseins (CN) and whey proteins (WP) that survived an *in vitro*, three-phase GI digestion, including simulated sequential gastric, duodenal and intestinal hydrolysis, have been investigated in a previous work (Picariello et al., 2010). The early extensive hydrolysis of CN, the partial resistance of β-lactoglobulin (β-Lg) to pepsin, and the resistance of a few specific protein regions were consistent with recent results regarding the degradation of milk proteins in piglets (Bouzerzour et al., 2012).

Although selected peptides may, in principle, exert their actions locally at the level of the GI tract, intestinal absorption prior to distribution to peripheral organs is generally an additional key factor that affects the bioavailability, kinetics and systemic actions of peptides *in vivo*. Peptides that are resistant to proteolytic degradation and are taken up to a significant extent are good candidates either to exert beneficial effects *in vivo* or, conversely, to act as

* Corresponding author at: Istituto di Scienze dell'Alimentazione (ISA), Consiglio Nazionale delle Ricerche (CNR), Via Roma 64, 83100 Avellino, Italy. Tel.: +39 0825 299216; fax: +39 0825 781585.
E-mail address: picariello@isa.cnr.it (G. Picariello).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.01.063

"immune sensitising factors", which are antigenic determinants that can induce sensitisation and elicit allergic reactions in predisposed individuals (Jensen-Jarolim & Untersmayr, 2006). Because the *in vivo* determination of intestinal uptake of nutrients or drugs in the intestine is technically difficult, transport studies across human colon adenocarcinoma (Caco-2) cell monolayers have been routinely utilised as an *in vitro* model to mimic absorption by the human intestinal epithelium. Despite their colorectal origin, differentiated Caco-2 cells maintain many morphological and functional traits that characterise the mature enterocytes of the small intestine, such as cell polarisation, microvillous structure, carrier-mediated transport systems, functional tight junctions between adjacent cells and expression of brush-border membrane (BBM)-associated proteases and peptidases. The intestinal absorption predicted by using Caco-2 cell monolayers is well correlated with intestinal uptake *in vivo,* at least from a qualitative standpoint (Artursson, Palm, & Luthman, 2012).

The transport of milk-derived peptides across Caco-2 cell monolayers has already been investigated using primarily single-peptide systems or simple mixtures of synthetic peptides (Cakir-Kiefer et al., 2011; Iwan et al., 2008; Regazzo et al., 2010; Sienkiewicz-Szłapka et al., 2009).

In the present work, the uptake by Caco-2 monolayers of the entire panel of peptides produced from simulated GI digestion of bovine CN and WP fractions has been evaluated. The peptides that translocated across the model epithelia were characterised by matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) and reversed phase-high performance liquid chromatography (RP-HPLC) coupled to either UV or electrospray (ESI) MS/MS detection. The peptides recovered from both the apical (UP) and basolateral (DOWN) insert media, along with the HPLC-isolated parent proteins and two proteolytically stable, synthetic peptides of β-Lg were assayed for their binding affinity to IgEs derived from the sera of $N = 5$ children diagnosed with cow's milk allergy (CMA).

2. Materials and methods

An individual bovine milk sample, which was obtained from a local farm, was preliminarily assessed by HPLC and MS as being heterozygous for both the β-CN A1 and A2 and β-Lg A and B and used for this study. After manual expression, the milk was immediately frozen and stored at −20 °C until use. Pepsin, trypsin, chymotrypsin, carboxypeptidase A, elastase, neutral red powder, dithiothreitol (DTT), iodoacetamide and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Ammonium bicarbonate, trifluoroacetic acid (TFA) and HPLC-grade solvents were purchased from Carlo Erba (Milan, Italy) and were used without any further purification.

2.1. Fractionation of milk proteins

CN and WP were separated from skim milk (20 mL) diluted two-fold with distilled water according to the Aschaffenburg method (Aschaffenburg & Drewry, 1959). The milk was acidified to pH 4.6 with 10% (v/v) acetic acid/1 M sodium acetate buffer, incubated for 30 min at 37 °C and centrifuged at 5000*g* at 4 °C for 10 min. The soluble WP were isolated by loading the supernatant onto Econo-pac® 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and the proteins were eluted with 50 mM ammonium bicarbonate. The column effluents were monitored by UV detection at $\lambda = 280$ nm (Ultrospec 2100 pro, Amersham Biosciences, Uppsala, Sweden). The WP was then quantitated by Bradford assay and freeze-dried. The CN precipitate was washed twice with sodium acetate/acetic acid buffer and then freeze-dried.

2.2. Gastro-pancreatic digestion

In vitro sequential, gastro-pancreatic digestion of milk proteins was performed as previously described (Picariello et al., 2010). Briefly, CN and WP were dissolved in 5% formic acid at a concentration of 1 mg/mL and incubated at 37 °C with pepsin (1:100 enzyme/protein ratio, w/w) for 60 min. Prior to pancreatic digestion, the samples were lyophilized and reconstituted twice with deionised water. The peptic digests were redissolved in 0.1 M sodium phosphate buffer (pH 7.0) at a concentration of approximately 1 mg/mL and further hydrolysed for 1 h at 37 °C with a pool of pancreatic enzymes containing trypsin (1:100, w/w), chymotrypsin (1:100, w/w), carboxypeptidase (1:100, w/w) and elastase (1:500, w/w). The simulated pancreatic digestion was stopped by heating the tubes in a boiling water bath for 5 min. The hydrolysis of milk proteins was monitored by RP-HPLC. The HPLC profiles of the digests, analyzed immediately before and after heating, did not appreciably differ, so excluding major heat-induced modifications of peptides.

Prior to application onto the Caco-2 monolayers, the gastro-pancreatic digests were desalted on Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) and then lyophilized after reconstitution with MilliQ water.

2.3. Synthetic peptides

β-Lg 125–135 and β-Lg 40–60, which were used as the GI-resistant model peptides, were synthesised by a solid-phase methodology using the Fmoc (9-fluorenylmethoxycarbonyl) strategy on a Pioneer peptide synthesizer (Synthesis System 9050 instrument, PE-Biosystems, Framingham, MA, USA). The purity (>95%) was assayed by both RP-HPLC and MALDI-TOF MS.

2.4. Cell culture

The Caco-2 cell line was obtained from ATCC (Philadelphia, PA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and Hank's Buffered Salt Solution (HBSS) were purchased from Lonza-BioWhittaker (Verviers, Belgium). The Caco-2 cells were cultured in DMEM supplemented with 20% heat-inactivated fetal calf serum (Gibco Life Technology), 1% nonessential amino acids (Euroclone), 2 mM L-glutamine and penicillin/streptomycin (Lonza-BioWhittaker). The Caco-2 cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The cells used in the experiments were between passages 20 and 30. Cells in log-phase growth were subcultured weekly by trypsinisation and were seeded at a ratio of 1:3 upon reaching 80% confluence. The culture medium was changed every 2–3 days. The cell viability was tested using the vital dye neutral red, according to the manufacturer's instructions.

2.5. Transepithelial transport studies

For the transport experiments, the cells were seeded at a density of 4.5×10^5 cells/cm^2 in 6-well filter support inserts with polyethylene terephthalate membranes (0.4 μm pore size, 23.1 mm diameter, 4.2 cm^2 growth surface area; purchased from BD Falcon, Italy). Cell attachment was improved by first covering the transwell membrane with type I bovine collagen (Gibco, Invitrogen, Italy) according to the manufacturer's instructions. The monolayers reached confluence 4 days after plating, and the cells differentiated for at least an additional 14 days prior to the transepithelial transport experiments. The integrity of the cell layers was evaluated by transepithelial electrical resistance (TEER) measurements