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A multidisciplinary investigation on the bioavailability and activity of peptides from lupin protein

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ABSTRACT

Lupin foods provide useful health benefits. Since tryptic and peptic peptides from lupin protein modulate cholesterol metabolism in HepG2 cells and increase LDL-uptake, this work had the goal of assessing whether these lupin peptides are absorbed by human intestinal Caco-2 cells. Cells were differentiated for 15 days and transport experiments were performed by incubating each lupin peptide mixture from the apical side. After 4 h, basolateral solutions were collected and analysed by HPLC-Chip-MS/MS. Eleven tryptic and eight peptic peptides, deriving from lupin storage proteins, were identified in the basolateral samples. An *in vitro* assay showed that basolateral peptides maintain their capacity to inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity and *in silico* docking simulations permitted to hypothesise which peptides may bind more efficiently to the HMGCoAR catalytic site. This is the first investigation providing evidence that lupin peptides with specific structures are potentially absorbed in human intestinal cells.

1. Introduction

The absorption of nutrients from the small intestinal lumen to blood circulation is mainly performed by enterocytes. An *in vitro* strategy to address the bioavailability problem consists of evaluating the uptake and transport of target compound(s) by human intestinal Caco-2 cells. When these cells are seeded in culture on polycarbonate filters, they undergo spontaneous differentiation leading to the formation of a polarised cell monolayer, coupled by tight junctions and expressing several morphological and functional features of small intestinal enterocytes (Sambuy et al., 2005). In this two-compartment system, the cell monolayer separates the apical (AP) compartment, corresponding to the intestinal lumen, from the basolateral (BL) one, corresponding to the intestinal vascular and lymphatic circulation (Ferruzza, Rossi, Scarino, & Sambuy, 2012; Sambuy et al., 2005).

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Abbreviations: ACE, angiotensin converting enzyme; AP, apical; APBS, adaptive Poisson–Boltzmann solver; BL, basolateral; DPP-IV, dipeptidyl peptidase IV; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDL, low density lipoprotein; MD, molecular dynamic; MLPInS, molecular lipophilic potential interaction score; PEP, prolyl endopeptidase; Plp95, pairwise linear potential; SREBP-2, regulatory element binding proteins 2; TEER, trans-epithelial electrical resistance

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There is now much interest for bioactive food peptides that may positively influence major body functions, once they are released by digestion from parental proteins and absorbed (Cam & de Mejia, 2012). The transport through intestinal cells is a major factor influencing their bioavailability. The hypothesis that small peptides may escape complete digestion and be transported from intestinal lumen into blood circulation is gaining acceptance, mainly due to numerous studies describing the *in vitro* trans-epithelial transport of bioactive peptides (Foltz et al., 2007; Miguel et al., 2008; Regazzo et al., 2010; Zhu et al., 2008), although its occurrence *in vivo* is still controversial (Miner-Williams, Stevens, & Moughan, 2014). Differentiated human intestinal Caco-2 cells express carrier-mediated transport systems for amino acids and di- and tri-peptides (Miguel et al., 2008; Thwaites, Brown, Hirst, & Simmons, 1993), show trans-cytotic activity (Heyman, Crain-Denoyelle, Nath, & Desjeux, 1990), and develop tight junctions involved in the paracellular passive route (Hashimoto & Shimizu, 1993; Hidalgo, Raub, & Borchardt, 1989). In particular, this model has been used for evaluating the bioavailability of antioxidant peptides from soybean β-conglycinin (Amigo-Benavent et al., 2014).

Lupin protein provides various health benefits (Arnoldi, Boschin, Zanoni, & Lammi, 2015; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008), particularly in the area of cholesterol reduction (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Sirtori et al., 2012), and hypertension (Arnoldi et al., 2015) or hyperglycaemia prevention (Duranti et al., 2008). We have recently demonstrated that tryptic and peptic peptides from lupin protein are able to interfere with 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity, up-regulating LDL-receptor and sterol regulatory element binding proteins 2 (SREBP-2), and increasing LDL-uptake in HepG2 cells (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014). Moreover, the same peptides inhibit the activity of angiotensin converting enzyme (ACE, EC 3.4.15.1) (Boschin, Scigliuolo, Resta, & Arnoldi, 2014a, 2014b), an effect possibly involved in the hypotensive effect observed *in vivo*.

From these findings the general question arose whether lupin peptides might be absorbed after food ingestion (Miner-Williams et al., 2014). As a first step to solve this issue, the present work was aimed at assessing whether any tryptic and peptic peptides from lupin protein may be absorbed by Caco-2 cells and transferred to the BL compartment and whether they maintain their hypocholesterolemic activity. The former objective was achieved by conducting transport experiments across differentiated human enterocytes and analysing BL solutions by HPLC-Chip-MS/MS, whereas the latter by using *in vitro* biological assays and bioinformatics tools.

2. Materials and methods

2.1. Chemicals and reagents

Formic acid, acetonitrile (ACN), sequence grade trypsin, and pepsin were from Sigma-Aldrich (Milan, Italy). LC-grade H_2O (18 MΩ cm) was prepared with a Milli-Q H_2O purification system (Millipore, Bedford, MA, USA). Centrifugal filter devices YM-30 (cutoff 30 kDa) were from Amicon Bioseparations (Millipore Corporation, Bedford, MA, USA). Dulbecco's Modified Eagle Medium (DMEM) was from GIBCO (Thermo Fisher Scientific, Waltham, MA USA). Foetal Bovine Serum (FBS) was from Hyclone Laboratories (Logan, UT, USA). Stable L-glutamine, 1% non-essential amino acids, and penicillin/streptomycin were from Euroclone (Milan, Italy). HMGCoAR assay Kit, red phenol, and PBS were from Sigma-Aldrich (St. Louis, MO, USA). Polycarbonate filters, 12 mm diameter, 0.4 μm pore diameter were from Transwell Corning Inc. (Lowell, MA, USA).

2.2. Preparation of the pepsin and trypsin peptide mixtures

The pepsin and trypsin hydrolysates from lupin protein are the same used in a previous paper investigating cholesterol metabolism modulation in HepG2 cells (Lammi et al., 2014). The procedure for protein extraction from seeds of *Lupinus albus* (cultivar Ares) and the conditions of protein hydrolysis are reported in detail there. In brief, proteins were extracted from defatted lupin flour with 100 mM Tris–HCl/0.5 M NaCl buffer, pH 8.2 for 2 h at 4 °C. After centrifugation at 6500 *g*, for 20 min at 4 °C, the supernatant was dialysed against 100 mM Tris–HCl buffer pH 8.2 for 24 h at 4 °C. After assessing the protein concentration by Bradford assay, the total protein extract was dissolved in Tris–HCl buffer 100 mM at pH 8, then the pH was adjusted to the optimal hydrolysis conditions for each enzyme (pH = 2 for pepsin and = 8 for trypsin) by adding 1 M NaOH or 1 M HCl. After 18 h incubation and enzyme inactivation, the mixtures were ultra-filtered through 3000 Da cut-off centrifuge filters (Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 12,000 *g* for 30 min at 4 °C. The peptide concentration in the permeates was measured according to a literature method (Levashov, Sutherland, Besenbacher, & Shipovskov, 2009), based on chelating the peptide bonds by Cu (II) in alkaline media and monitoring the change of absorbance at 330 nm. In brief, the reagent contained 0.6 M sodium citrate, 0.9 M sodium carbonate, and 0.07 M copper sulfate, 2.4 M NaOH at pH 10.6. A solution containing X μL peptide mixture, (500 – X) μL water, 500 μL 6% (w/w) NaOH in water, and 50 μL active reagent was prepared. The reaction was carefully mixed, incubated for 15 min at 20 °C, and then the optical density of the solution was measured at 330 nm. A sterile solution of peptone from casein at 10 mg/mL in water was used as standard for the calibration curve; the assay was linear in the range 100–1000 μg of peptides in cuvette.

2.3. Cell culture and differentiation

Caco-2 cells, obtained from INSERM (Paris, France), were sub-cultured at low density (Natoli et al., 2011). Cells were routinely sub-cultured at 50% density and maintained at 37 °C in a 90%/10% air/CO_2 atmosphere in DMEM containing 25 mM glucose, 3.7 g/L $NaHCO_3$, 4 mM stable L-glutamine, 1% non-essential amino acids, 100 U/L penicillin, 100 μg/L streptomycin (complete medium), supplemented with 10% heat inactivated foetal bovine serum (FBS) (Hyclone Laboratories). For differentiation, cells were seeded on polycarbonate filters, 12 mm diameter, 0.4 μm pore diameter (Transwell Corning Inc.) at a 3.5×10^5 cells/cm^2 density in complete medium supplemented with 10% FBS in both AP and BL compartments for 2 days to allow the formation of a confluent cell monolayer. Starting from day 3 after seeding, cells