Antioxidant activity and protective effects of peptide lunasin against oxidative stress in intestinal Caco-2 cells

María José García-Nebot, Isidra Recio, Blanca Hernández-Ledesma*

Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM, CEI UAM+CSIC). Nicolás Cabrera, 9. 28049 Madrid, Spain

* Corresponding author: B. Hernández-Ledesma
Nicolás Cabrera, 9. 28049 Madrid, Spain
Phone: +34 910017970
Fax : +34 910017905
e-mail: b.hernandez@csic.es

Abstract

Oxidative stress is one of the most critical factors implicated in many disorders of the gastrointestinal tract, such as inflammatory bowel diseases and colon cancer. Lunasin is a seed peptide known by its properties against cancer and cardiovascular diseases. The present study investigated the *in vitro* antioxidant activity of this peptide that demonstrated to be a potent scavenger of peroxyl and superoxide radicals. In addition, the protective role of lunasin on cell viability and antioxidant defenses of human Caco-2 cells challenged by hydrogen peroxide and *tert*-butylhydroperoxide was evaluated. This peptide remained partially intact during incubation time with cells, preventing of the oxidative damage induced by both chemical agents. The direct antioxidant action of lunasin (within the physiological range of concentrations) on enterocytes exposed to oxidizing species makes this peptide a promising agent to preserve the integrity of intestinal mucosa against oxidative damage related diseases.

Keywords: Peptide lunasin, antioxidant, oxidative stress, intestinal health

1. Introduction

During cell metabolism, oxygen is partially reduced to reactive oxygen species (ROS). These species play a physiological role in cellular processes, including proliferation, cell cycle and death, and signal transduction (Martindale and Holbrook, 2002; Owuor and Kong, 2002). However, their high reactivity makes ROS being responsible for oxidation of lipid, proteins and DNA causing damaging effects on vital tissues. Mammalian cells express detoxifying or radical scavenging systems that counteract the destructive effects of ROS. Nevertheless, high levels of these species caused by an imbalance between their production and the ability of the antioxidant defences to detoxify the organism provoke a status of oxidative stress. This status and its subsequent damages to vital cellular components have been associated with numerous severe degenerative diseases (Carocho and Ferreira, 2013).

The intestine is continuously exposed to reactive species endogenously generated from luminal contents as well as to oxidants daily ingested with the foods (Couto et al., 2012). A large literature exists on the pivotal role that ROS play in the physiology and pathology of the intestine as well as in the microbiota profile (Pavlick et al., 2002; Graham Espey, 2013). Moreover, many disorders of the gastrointestinal tract, such as inflammatory bowel diseases and colon cancer are associated with increased levels of ROS and alterations in the redox status of intestinal cells (Udilova et al., 2003; Wijeratne and Cuppett, 2007; Rezaie et al., 2007).

In spite of its remarkable effectiveness, the endogenous antioxidant systems are not sufficient, and humans, in order to maintain ROS concentrations at low levels, are dependent on antioxidants present in the diet (Pietta, 2000). A number of natural dietary antioxidants have been revealed as potential preventative/therapeutic agents against oxidative stress (Carocho and Ferreira, 2013). Among them, food-derived peptides have attracted the attention because of the large evidence on their *in vitro* antioxidant properties (Power et al., 2013). In

addition to their potential as safer alternatives to synthetic antioxidants used to prevent oxidative reactions in foods, antioxidant peptides can also act reducing the risk of numerous oxidative stress-associated diseases (Meisel, 2004). Furthermore, peptides can act synergistically with non-peptide antioxidant enhancing their protective effect (Kitts and Weiler, 2003; Hernández-Ledesma et al., 2007).

Lunasin is a 43-amino acid peptide identified in soybean and other plants (Gálvez and de Lumen, 1999). It has been demonstrated to be present in all the soybean varieties analyzed to date, at concentrations ranged from 4.4 to 70.5 mg lunasin/g of soy protein (Hernández-Ledesma et al., 2009a). Daily consumption of 25 g of soy protein, recommended by Food and Drug Administration (FDA) to reduce coronary disease (FDA, 1999), supplies lunasin in quantity ranged from 110 mg (21 μmol) to 1760 mg (350 μmol). It is known that lunasin is protected by the soybean Bowman-Birk proteases inhibitor (BBI) against the action of gastric and pancreatic enzymes (Hernández-Ledesma et al., 2009b). Therefore, high percentage of daily ingested peptide remains intact during its passage through the gastrointestinal tract, reaching target organs and tissues in an intact and active form (Hsieh et al., 2010). It has been demonstrated, by both cell culture and animal models, that this peptide exerts potent chemopreventive properties against several types of cancer, such as breast, colon, and prostate cancer, acting through different cellular and molecular pathways (see review of Hernández-Ledesma et al., 2013). Moreover, this peptide is already commercialized in the US by its reported effects on cardiovascular system as reducing agent of low density lipoprotein (LDL) cholesterol concentration (Gálvez, 2012). Preliminary studies have suggested that lunasin exerts antioxidant and anti-inflammatory properties that might contribute on its physiological properties (Hernández-Ledesma et al., 2009c; Cam et al., 2013). However, to date, no information about the direct protective effects of this peptide against cellular oxidative stress status have been reported.

Because of the directly interaction between food and intestine, nowadays, it exists a great interest to evaluate the capacity of food antioxidants to protect intestinal epithelium against oxidative stress conditions, and subsequently against the development of intestinal pathologies. The present paper aimed to assess the *in vitro* antioxidant activity of peptide lunasin through radical scavenging and ferrous ion chelating activities. Also, the protective effects of this peptide against the oxidative damage to intestinal mucosa were evaluated, using differentiated human Caco-2 cells as a model of the human intestine, and two chemical agents, hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-BOOH) as inducing agents.

2. Materials and Methods

2.1. Reagents

Peptide lunasin was synthesized by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China). Fluorescein disodium (FL), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), β-nicotinamide adenine dinucleotide (NADH), superoxide dismutase (SOD), ethylenediaminetetraacetic acid (EDTA), ferrozine, trifluoroacetic acid (TFA), 3-[4,5-dimethylthiazol-2-yl]-2,3-diphenyl tetrazolium bromide (MTT), dichlorofluorescin (DCFH), and dihydrorhodamine (DHR) were purchased from Sigma Chemical (St. Louis, MO, USA). 2,2'-azobis (2-methylpropionamide)-dihydrochloride (AAPH), and 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were from Aldrich (Milwaukee, WI, USA). The rest of chemicals used were of HPLC grade.

2.2. In vitro antioxidant activity

2.2.1. Oxygen radical absorbance capacity assay

An oxygen radical absorbance capacity (ORAC)-FL assay was used based on that optimized for protein hydrolyzates and peptides by Hernández-Ledesma et al. (2005). Briefly, the reaction was carried out at 40 ºC in 75 mM phosphate buffer (pH 7.4). The final assay

mixture (200 μL) contained FL (70 nM), AAPH (14 mM) and antioxidant [Trolox (0.2-1.6 nmol) or sample (at different concentrations)]. Fluorescence was recorded during 137 min (104 cycles) in a FLUOstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) with 485 nm excitation and 520 nm emission filters. The equipment was controlled by the FLUOstar Control ver. 1.32 R2 software for fluorescence measurement. Three independent runs were performed for each sample. Final ORAC-FL value was expressed as μmol Trolox equivalent/μM peptide.

2.2.2. Superoxide anion scavenging activity

Measurement of superoxide anion scavenging activity of lunasin was investigated according to the method reported by Li et al. (2011) and optimized in our laboratory for its use in microplates. The reaction was carried in 0.1 M phosphate buffer (pH 7.4) with a final mixture volume of 200 μL that contained PMS (60 μM, 50 μL), NBT (156 μM, 50 μL), NADH (468 μM, 50 μL), and antioxidant [SOD (0.078-40 μM) or lunasin (0.15-20 μM)]. 96-well microplates (Nunc maxisorp F96, Roskilde, Denmark) were used. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured on a Varioskan Flash microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

The capability of scavenging to superoxide radical was calculated using the following equation:

$$\text{Scavenging capacity (\%)} = (A1 - A2) \times 100/(A3 - A2)$$

where A1 is the absorbance of the sample, A2 is the absorbance of the blank and A3 is the absorbance of the control.

Three independent runs were performed for each sample. The SOD or lunasin concentration (μM) needed to scavenge 50% of superoxide anion radicals (IC_{50} value) was determined using the software GraphPadPrism v.4.01 (GraphPad, La Jolla, CA, USA).

2.2.3. Ferrous ion chelating activity

The ferrous ion chelating activity assay was performed according to Decker and Welch (1990) with some modifications, and using EDTA as standard. The reaction mixture containing 1 mL of 100 mM sodium acetate buffer (pH 4.9), 100 μL of 181.4 μM $FeCl_2.4H_2O$, and 100 μL of antioxidant [EDTA or lunasin (1.25-80 μM)] was incubated at 25 °C for 5 min. After this time, 50 μL of ferrozine solution (40 mM) were added and the mixture was gently shaken. A volume of 200 μL of the mixture was placed in 96-well microplates (Nunc maxisorp F96) and the absorbance was read at 562 nm on the Varioskan Flash microplate reader (Thermo Fisher Scientific).

The capability of metal chelating was calculated using the following equation:

$$\text{Chelating capacity (\%)} = (A1 - A2) \times 100/A3$$

where A1 is the absorbance of the sample, A2 is the absorbance of the blank and A3 is the absorbance of the control.

Three independent runs were performed for each sample. The concentration (μM) of EDTA or lunasin needed to reduce 50% ferrous ions (IC_{50} value) was determined using the software GraphPadPrism v.4.01 (GraphPad).

2.3. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (HTB-38, Rockville, MD, USA), and were used between passages 30 and 40. The cells were maintained in 75 cm^2 flaks in Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical) with 10% (v/v) fetal bovine serum (FBS, Sigma Chemical), 1% (v/v) nonessential amino acids (Lonza Group Ltd., Basel, Switzerland), and 1% (v/v) antibiotic solution with fungizone (Lonza Group Ltd.) at pH 7.2-7.4. The cells were maintained at 37ºC in an incubator under a 5% CO_2/95% air at constant humidity. Culture medium was changed every 2 days.

The cells at 70% confluence were detached with trypsin-EDTA (2.5 g/L trypsin, 0.2 g/L EDTA), harvested from the flask, and suspended in culture medium. They were seeded onto polyester membrane chamber inserts (12 mm diameter, 0.4 μm pore size; Transwell®, Corning Costar Corp., Corning, NY, USA) at a density of 5×10^4 cells/cm^2, with 1 mL of medium in the basolateral chamber and 0.75 mL of suspended cells in the apical chamber. Seven to nine days after the initial seeding, the integrity of the monolayer of differentiated cells was monitored by measuring the transepithelial electrical resistance (TEER) value. Cells with values higher than 400 Ω x cm^2 were used in the assays.

2.4. Stability of lunasin

Caco-2 cells were seeded onto 6-well plates (Corning Costar Corp.) at a density of 5×10^4 cells/cm^2. The culture medium was changed every two days, and at seven to nine days from initial seeding, the culture medium was aspirated, and cell monolayers were washed with phosphate buffer saline (PBS). Then, cell cultures were treated with 2.5 μM lunasin and incubated at 37ºC for 24 h, taking supernatant samples at 0, 6, 16, and 24 h of incubation.

To analyze the relative amount of lunasin remaining in the culture medium after treatment with this peptide, the supernatants were subjected to RP-HPLC coupled to tandem mass spectrometry (RP-HPLC-MS/MS) analysis on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source as previously described (Silveira et al., 2013). The column used was a Mediterranea Sea_{18} (150 x 2.1 mm, Teknokroma, Barcelona, Spain), the injection volume was 50 μL, and the flow was set at 0.2 mL/min. Peptides were eluted with a linear gradient of solvent B (ACN:trifluoroacetic acid (TFA) 1000:0.27 v/v) in A (water:TFA 1000:0.37 v/v) going from 0% to 45% in 130 min. The *m/z* spectral data were processed and transformed to representing

mass values, and the peak area corresponding to lunasin and/or its derived fragments were integrated Using Data Analysis™ (version 4.0; Bruker Daltonics).

2.5. Cell treatment conditions

Cells were incubated for 24 h with various concentrations of peptide lunasin, ranged from 0.5 to 25 μM. To evaluate both direct and protective effects against oxidative stress, the incubation period was followed by a 3 h treatment with culture medium (direct effect) or oxidant chemicals H_2O_2 (4 mM) or *t*-BOOH (3 mM). In both cases, different biomarkers were evaluated.

2.5.1. Cell viability

Cell viability was determined using the MTT assay. It is based on the reduction of the tetrazolium ring of MTT by mitochondrial dehydrogenases, yielding a blue formazan product, which can be measured spectrophotometrically. Caco-2 cells were seeded onto 48-well plates (VWR International, Radnor, PA, USA) at a density of 5×10^4 cells/cm^2. After seven to nine days from initial seeding, cell monolayers were washed with PBS, treated with lunasin, and incubated overnight. Afterwards, culture medium was removed, cells were washed with PBS and incubated with medium or chemical oxidants at 37 ºC for 1.5 and 3 h. MTT solution (0.5 mg/mL final concentration) was added to each well, and the cells were incubated for 2 h at 37 ºC. The supernatant was aspirated and insoluble formazan crystals formed were dissolved in dimetilsulfoxide:ethanol (1:1), measuring the absorbance at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech).

The results were expressed as percentage of the control, considered as 100%. Experiments were carried out in triplicate, and the results were expressed as the mean ± standard deviation (SD).

2.5.2. Intracellular ROS levels

2.5.2.1. Measurement of ROS by fluorescence

Intracellular ROS levels were quantified following the method described by Alía et al. (2005), using DCFH as fluorescent probe. Caco-2 cells were treated with lunasin as mentioned above. After 23.5 h incubation with the peptide, a solution of DCFH solution was added to each well (5 μM final concentration) and cells were incubated for 30 min at 37 ºC. Then, the cells were incubated with culture medium (direct effects) or subjected to chemical-induced oxidative stress with H_2O_2 or *t*-BOOH (protective effects) for 1.5 or 3 h. Afterwards, the fluorescence intensity was measured at $\lambda_{excitation}$ and $\lambda_{emission}$ of 485 nm and 530 nm, respectively, in a FLUOstar OPTIMA plate reader (BMG Labtech). The results were expressed as percentage of the control, considered as 100%. The assay was run in triplicate, and the results were expressed as the mean ± SD.

2.5.2.2. Measurement of ROS by flow cytometry

The intracellular accumulation of ROS in Caco-2 cells under chemical inducing conditions was also evaluated by flow cytometry using DHR, which is oxidized to fluorescent rhodamine (Cilla et al., 2008). Caco-2 cells were treated with lunasin as above mentioned. Then, the culture medium was removed, cell monolayers were washed with PBS, and resuspended in a trypsin-EDTA solution. After centrifuging the cells at 1200 rpm for 5 min, they were dissolved in 0.5 mL of DHR (final concentration 5 μM), and incubated for 30 min at 37 ºC in darkness. Fluorescent intensity was determined by flow cytometry at $\lambda_{excitation}$ and $\lambda_{emission}$ of 488 nm 525 nm, respectively in a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The results were analyzed using the software Kaluza Flow Cytometry Analysis Version 1.2 (Beckman Coulter Inc., Brea, CA, USA). The data were obtained from the six replicates, and the results were expressed as the mean ± standard

deviation (SD). Control cells were used throughout each assay. At least 5,000 cells per sample were analyzed.

2.8. Statistical analysis

Data were analyzed by a one-way ANOVA, followed by the Tukey's test. Statgraphics Plus v.5.1 software (Rockville, MD, USA) was used to find significant differences ($p<0.05$)

3. Results and Discussion

3.1. In vitro antioxidant capacity of lunasin

The antioxidant capacity of lunasin was assessed through its ability to scavenge peroxyl and superoxide radicals, as well as its ferrous ion chelating activity. Figure 1a and 1b shows the concentration (μmol in the assay) ranges that ensured linearity between the net area under the curve (Net AUC) and the concentration of antioxidant (Trolox or lunasin). To determine the ORAC value of lunasin, the slope of the net AUC versus concentration curve was divided by the slope of the Trolox calibration curve. The ORAC value for lunasin was of 3.44 ± 0.07 μmol Trolox equivalents/μmol lunasin. This activity was higher than that of natural antioxidants such as vitamin C (1.65 μmol Trolox equivalents/μmol vitamin C) (Huang et al., 2010), or synthetic antioxidants such as butylated hydroxyanisole (BHA) (2.43 μmol Trolox equivalents/μmol BHA) (Dávalos et al., 2004). As shown in the ORAC database prepared by Li and Li (2013), length of peptides derived from food sources with peroxyl radical scavenging activity was from 4 to 20 amino acids. To our knowledge, lunasin, with 43 amino acids, is the first peptide containing more than 20 amino acids that shows this activity. Potent activity of lunasin was attributed to the presence of amino acids Trp, Cys, and Met in its sequence (Hernández-Ledesma et al., 2005). These amino acids were also suggested to be

responsible for the ABTS radical scavenging activity reported for this peptide (Hernández-Ledesma et al., 2009c).

In the present study, it was also demonstrated the capacity of lunasin to neutralize superoxide radicals. The effect was dose-dependent at concentrations ranged from 0.15 to 5 μM, reaching 61.6% neutralization at 5 μM, and maintaining a constant neutralization value at higher concentrations (data not shown). The effect was compared to that of enzyme SOD that reached 75.0% neutralization at 10 μM. In the linear range, the IC_{50} or concentration of antioxidant needed to neutralize 50% radicals was calculated, and values of 0.85 μM and 2.34 μM were obtained for lunasin and SOD, respectively. The ability of lunasin to chelate ferrous ions was also evaluated. At the maximum concentration used in the assay (80 μM), lunasin only chelated 23.9% of these ions, while EDTA used as standard was able to chelate 100% ions at 10 μM. However, the study carried out by Jeong et al. (2010) showed that lunasin purified from *Solanum nigrum* L. chelated ferrous ions by 73% at 10 μM. Discrepancy with our results could be due to the different origin of lunasin. In our study, synthetic lunasin according to the sequence identified in soybean demonstrated potent antioxidant properties through scavenging of free radicals.

3.2. Stability of lunasin

In order to evaluate the protective effects of lunasin against oxidative stress, it was initially needed to study the behavior of this peptide after its addition to Caco-2 cells. It has been demonstrated that these cells undergo a process of differentiation leading to the formation of a monolayer of cells, expressing multiple morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005). The first objective was to assess the resistance of this peptide to brush-border membrane peptidases expressed in Caco-2 cells after its addition to DMEM. For that, the medium without and with lunasin (2.5 μM) at

283 different incubation hours (0, 6, 16, and 24 h) was analyzed by HPLC-MS/MS. It was
284 observed that, in addition to other components present in the medium, lunasin was clearly
285 visible along the incubation time. As an example, Figure 2a shows the chromatogram
286 obtained after 16 h-incubation of cells without and with lunasin. The lunasin's peak area was
287 measured (Figure 2b), and a decrease in content of this peptide with the incubation time was
288 observed, reaching 12% reduction of this peptide after 16 h compared to the content measured
289 at the starting incubation point. The rest of lunasin (88%) remained intact in the medium and
290 no derived fragments were identified. These results suggest the possibility of lunasin's
291 internalization into human intestinal Caco-2 cells. If this fact is confirmed, lunasin might
292 exert a potential protective role inside and outside the cell. Preliminary studies carried out
293 with mouse C3H10T1/2 and NIH3T3 fibroblast cells demonstrated that lunasin was
294 internalized by these cells (Galvez et al., 2001). Moreover, a recent study has reported the
295 internalization of lunasin into human macrophages that is amplified when these cells are in an
296 inflammatory state (Cam et al., 2013).
297
298 *3.3. Cell viability*
299 The direct effects of lunasin on Caco-2 cells viability were evaluated using the MTT
300 assay. This assay provides a sensitive measurement of the cell metabolic status, specifically of
301 the mitochondria, which displays early cellular redox changes (Circu and Aw, 2010).
302 Treatment of Caco-2 cells for 24 h with lunasin evoked no changes in cell viability, indicating
303 that the concentrations selected for the study (0.5-25 μM) did not damage cell integrity during
304 the period of incubation. As mentioned above, Caco-2 cell line, originally derived from
305 human colon carcinoma, can spontaneously differentiate into intestinal epithelium under
306 standard culture conditions and the differentiated cells express enterocyte-like features. These
307 results are in agreement with previous studies that indicated that lunasin does not affect

viability of non-transformed cells while viability of transformed cells by chemical agents or oncogenes, and tumorigenic cells is notably affected by this peptide (Lam et al., 2003; Galvez et al., 2011).

To study the protective effects of lunasin against chemical-induced oxidative damage in Caco-2 cell monolayers, they were pre-incubated with lunasin for 24 h, exposed to H_2O_2 or *t*-BOOH for 1.5 and 3 h, and then, cell viability was measured by the MTT assay. As shown in Figure 3a, treatment of Caco-2 cells with H_2O_2 (4 mM) for 1.5 h provoked a significant reduction of cell viability of 30%, compared to non-stimulated cells. Longer treatment (3 h) with this chemical did not result in a higher reduction of cell viability (data not shown). Previous studies had also reported significant decreases of cell viability after treatment of Caco-2 cells with 5 mM H_2O_2 for 2 h (Laparra et al., 2008; García-Nebot et al., 2011) or concentrations of this chemical in the range from 0.3 to 10 mM for 20 h (Yokomizo and Moriwaki, 2006). In the case of *t*-BOOH (3 mM), treatment for 1.5 h resulted in 20% reduction of Caco-2 cells viability (Figure 3b). However, previous studies had observed significant reductions at longer incubation time (6 h) using concentrations of this chemical of 0.1 mM and 4 mM (Manna et al., 2005; Kim et al., 2013).

Pretreatment of Caco-2 cells with lunasin before induction with H_2O_2 for 1.5 h protected cells from the effects of this chemical. As shown in Figure 3a, the percentage of viable cells treated with lunasin at concentrations of 1-25 μM before addition of H_2O_2 was significantly higher to that observed for non-treated cells, being similar to that observed for non-stressed cells. However, for Caco-2 cells stimulated with *t*-BOOH, only the pretreatment with 1 μM lunasin resulted in a significant increase of viable cells up to levels of non-stressed cells (Figure 3b). Higher lunasin's concentrations also increased the percentage of viable cells but the final values were not significantly different from those obtained for stressed-cells. Moreover, lunasin did not protect cells from the effects of *t*-BOOH when this chemical was

incubated for 3 h (data not shown). A higher damaging effect on viability was observed when Caco-2 cells were challenged with H_2O_2 than with *t*-BOOH, being lunasin able to exert more protective actions. In the last years, milk protein-derived hydrolyzates have demonstrated to preserve viability and protect against oxidative damage in different cell lines stimulated with H_2O_2. In particular, caseinophosphopeptides have demonstrated to exert this effect in H_2O_2-induced Caco-2 cells (García-Nebot et al., 2011) while casein and whey protein hydrolyzates do it in H_2O_2-induced hepatic HepG2 and neuronal PC12 cells, respectively (Xie et al., 2013; Zhang et al., 2012).

3.4. Intracellular ROS levels

At gastrointestinal level, increased levels of ROS have been directly associated to inflammatory bowel diseases and colon cancer (Udilova et al., 2003; Wijeratne and Cuppett, 2007; Rezaie et al., 2007). Direct evaluation of intracellular ROS is a good indicator of the oxidative damage to living cells (Wang and Joseph, 1999). In our study, measurement of the intracellular ROS levels was carried out using two fluorescent probes, DCFH and DHR. When applied to intact cells, the nonionic, nonpolar DCFH crosses cell membranes and in the presence of ROS, it is oxidized to highly fluorescent dichlorofluorescein (DCF) (LeBel et al., 1992). This probe has better use as a marker of the overall oxidative stress in cells than as indicator of the formation of H_2O_2 or other ROS. As shown in Figure 4a, lunasin did not increase ROS production in non-stressed Caco-2 cells. However, we found that 4 mM H_2O_2 or 3 mM *t*-BOOH challenge for 1.5 h significantly increased ROS levels (untreated cells 100.0 ± 1.2; treated cells with H_2O_2 348.3 ± 18.0; treated cells with *t*-BOOH 479.1 ± 35.4) ($p<0.05$). The lower effect observed on ROS levels resulting from H_2O_2 challenge could be due to the poor activity of this agent on DCFH oxidation in such short induction time. It has been previously reported that H_2O_2 alone does not oxidize DCFH, requiring its previous

transformation to other ROS with higher oxidant capacity (Royall and Ischiropoulos, 1993). Other possible explanation of the higher degree of oxidative damage provoked by *t*-BOOH might be the higher stability of this chemical in aqueous solution than that of H_2O_2, permitting *t*-BOOH a longer and more sustained effect on the cell (Alía et al., 2005). The pre-treatment with lunasin for 24 h significantly neutralized the ROS-generating ability of both chemicals by Caco-2 cells (Figure 4a). In the case of H_2O_2-stressed cells, the highest protection was observed at lunasin concentration of 0.5 μM that reduced ROS levels by 32.9% compared to non-lunasin treated cells ($p<0.05$). At higher lunasin concentrations, no further reduction was observed. However, for *t*-BOOH stressed cells, dose-dependent effects of lunasin on ROS levels were determined, reaching 62.7% reduction at 25 μM ($p<0.05$).

The second probe was DHR, a non-fluorescent molecule that, by oxidation, yields rhodamine 123, a cationic and lipophilic fluorescent probe which levels were measured with flow cytometry. As shown in Figure 4b, both chemicals induced a notable increase of ROS levels (without H_2O_2 100.0 ± 11.8; with H_2O_2 131.2 ± 11.7; with *t*-BOOH 162.8 ± 10.6). These results confirm the higher capacity of *t*-BOOH to induce oxidative stress in Caco-2 cells than that shown by H_2O_2. Pre-treatment with lunasin resulted in an important reduction of ROS levels. In Caco-2 cells stressed with both chemicals, the maximum effect was observed at lunasin concentration of 1 μM, reaching 54.4% of reduction in H_2O_2-stressed cells and 27.1% in *t*-BOOH-stressed cells, compared to non-lunasin treated cells (Figure 4b). In contrast to the effects observed with DCFH, when using DHR, most potent effects were observed when Caco-2 cells were pre-incubated with lunasin before H_2O_2 challenge, in which ROS levels were decreased up to levels lower than those measured in non-challenged cells. The differences between both fluorescent probes could be due to the molecular basis of the determinations or to the singular specificity of each probe. Halliwell and Whiteman (2004) and Gomes et al. (2005) have demonstrated that DHR is a probe widely used to detect several

ROS, such as $OH^{\bullet}$, $ONOO^{-}$, or NO_2^{-}, but is poorly responsive to $O_2^{\bullet-}$, H_2O_2 or $NO^{\bullet}$. Moreover, the differences found with DCFH and DHR might reflect the different molecular basis of these determinations.

The protective effects of lunasin on intestinal cells against oxidative damage reducing ROS production is in agreement with previous results of this study indicating that this peptide is an effective scavenger of superoxide and peroxyl radicals *in vitro*. To date, few peptides have demonstrated to exert protective effects against oxidative damage at intestinal level. Recently, García-Nebot et al., (2011) revealed this ability for caseinophosphopeptides obtained after digestion of casein under simulated gastrointestinal digestion.

4. Conclusions

In summary, our results have demonstrated that physiological doses of lunasin exert a protective effect in Caco-2 cells against H_2O_2 and *t*-BOOH-induced oxidative stress and subsequent cell death. This effect it mainly produced through its ability to scavenge ROS. Our results point out for the first time a direct antioxidant action of this peptide on enterocytes exposed to oxidizing species. As intestinal concentration of lunasin might be much higher than serum concentration, these data have an interesting biological significance, suggesting the possibility that this peptide may significantly contribute to preserve the integrity of intestinal mucosa against oxidative damage related disorders. Moreover, these results open the door to future studies on the antioxidant activity of lunasin in other cell lines that contribute to its already demonstrated properties as chemopreventive and cardioprotective agent.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work has received financial support from projects AGL2011-24643, Consolider Ingenio 2010 FUN-C-Food CSD2007-00063, P2009/AGR-1469, FEDER-INNTERCONECTA-GALICIA (ENVELLEFUN) and FP7-SME-2012-315349 (FOFIND). The authors are participant in the FA1005COST Action INFOGEST on food digestion. B.H.-L. acknowledges Ministry of Economy and Competitiveness for her "Ramón y Cajal" contract.

Figure captions

Figure 1. *In vitro* antioxidant capacity of lunasin measured by ORAC assay: Linear regression of the net area under the curve (net AUC) vs concentration of (a) Trolox, (b) Lunasin in the assay. Results are expressed as means of three independent runs.

Figure 2. Stability of peptide lunasin in DMEM medium added to Caco-2 cells. (a) UV-chromatogram obtained after 16 h-incubation of Caco-2 cells without and with lunasin at 2.5 μM. (b) Relative amount of lunasin at different incubation hours (0, 6, 16, and 24 h). Different superscript letters (a, b) denote statistically significant differences ($p<0.05$).

Figure 3. Protective effects of lunasin on cell viability. Caco-2 cells were pre-incubated with lunasin (concentration ranged from 0.5 to 25 μM) for 24 h before treatment with medium (control cells) or medium supplemented with chemicals (a) hydrogen peroxide (H_2O_2, 4 mM) and (b) *tert*-butyl hydroperoxide (*t*-BOOH, 3 mM) for 1.5 h, and cell viability was measured by the MTT assay. Different superscript letters (a-c) denote statistically significant differences ($p<0.05$).

Figure 4. Direct and protective effects of lunasin against intracellular ROS generation. Caco-2 cells were pre-incubated with lunasin (concentration ranged from 0.5 to 25 μM) for 24 h before treatment with medium (control cells) or medium supplemented with hydrogen peroxide (H_2O_2, 4 mM) and *tert*-butyl hydroperoxide (*t*-BOOH, 3 mM) for 1.5 h, and the intracellular ROS levels were measured using DCFH as fluorescent probe. Different superscript letters (a-c) denote statistically significant differences ($p<0.05$).

Figure 5. Direct and protective effects of lunasin against intracellular ROS generation. Caco-2 cells were pre-incubated with lunasin (concentration ranged from 0.5 to 25 μM) for 24 h before treatment with medium (untreated cells) or medium supplemented with hydrogen peroxide (H_2O_2, 4 mM) and *tert*-butyl hydroperoxide (*t*-BOOH, 3 mM) for 1.5 h, and the intracellular ROS levels were measured using DHR as fluorescent probe. Different superscript letters (a-c) denote statistically significant differences ($p<0.05$).

447 **References**

448 - Alía, M., Ramos, S., Mateos, R., Bravo, L., Goya, L., 2005. Response of the antioxidant
449 defense system to tert-Butyl hydroperoxide and hydrogen peroxide in a human hepatoma cell
450 line (HepG2). J. Biochem. Mol. Toxicol. 19, 119-128.
451 - Cam, A., Sivaguru, M., González de Mejia, E., 2013. Endocytic mechanism of
452 internalization of dietary Peptide lunasin into macrophages in inflammatory condition
453 associated with cardiovascular disease. PLoS One 8, e72115.
454 - Carocho, M., Ferreira, I. C. F. R., 2013. A review on antioxidants, prooxidants and related
455 controversy: Natural and synthetic compounds, screening and analysis methodologies and
456 future perspectives. Food Chem. Toxicol. 51, 15-25.
457 - Cilla, A., Laparra, J. M., Alegría, A., Barberá, R., Farré, R., 2008. Antioxidant effect derived
458 from bioaccessible fractions of fruit beverages against H_2O_2-induced oxidative stress in Caco-
459 2 cells. Food Chem. 106, 1180-1187.
460 - Circu, M. L., Aw, T. Y., 2010. Reactive oxygen species, cellular redox systems, and
461 apoptosis. Free Rad. Biol. Med. 48, 749-762.
462 - Couto M. R., Gonçalves, P., Catarino, T., Araújo, J. R., Correia-Branco, A., Martel, F.,
463 2012. The effect of oxidative stress upon the intestinal uptake of folic acid: *in vitro* studies
464 with Caco-2 cells. Cell Biol. Toxicol. 28, 369-381.
465 - Dávalos, A., Gómez-Cordovés, C., Bartolomé, B., 2004. Extending applicability of the
466 oxygen radical absorbance capacity (ORAC Fluorescein) assay. J. Agric. Food Chem.52, 48-
467 54.
468 - Decker, E. A., Welch, B., 1990. Role of ferritin as a lipid oxidation catalyst in muscle food.
469 J. Agric. Food Chem. 38, 674-677.

- FDA Talk Paper. FDA approves new health claim for soy protein and coronary heart disease. FDA, United States Department of Health and Human Services, October 26, 1999. Washington, DC: United States Government Printing Office, 1999.

- Gálvez, A. F., 2012. Abstract 10693: Identification of lunasin as the active component in soy protein responsible for reducing LDL cholesterol and risk of cardiovascular disease. Circulation 126, A10693.

- Galvez, A. F., Chen, N., Macasieb, J., de Lumen, B. O., 2001. Chemopreventive property of a soybean peptide (Lunasin) that binds to deacetylated histones and inhibit acetylation. Cancer Res. 61, 7473-7478.

- Gálvez, A. F., de Lumen, B. O., 1999. A soybean cDNA encoding a chromatin binding peptide inhibits mitosis of mammalian cells. Nat. Biotechnol. 17, 495-500.

- Galvez, A. F., Liping, H., Magbanua, M. M. J., Dawson, K., Rodriguez, R. L., 2011. Differential expression of thrombosponding (THBS1) in tumorigenic and nontumorigenic prostate epithelial cells in response to a chromatin-binding soy peptide. Nutr. Cancer 63, 623-636.

- García-Nebot, M. J., Cilla, A., Alegría, A., Barberá R., 2011. Caseinophosphopeptides exert partial and site-specific cytoprotection against H_2O_2-induced oxidative stress in Caco-2 cells. Food Chem. 129, 1495-1503.

- Gomes, A., Fernandes, E., Lima, J. L. F. C., (2005). Fluorescence probes used for detection of reactive oxygen species. J. Biochem. Biophys. Methods 65, 45-80.

- Graham Espey, M., 2013. Role of oxygen gradients in shaping redox relationships between the human intestine and its microbiota. Free Rad. Biol. Med. 55, 130-140.

- Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage *in vivo* and in cell culture: how should you do it and what do the results mean? Brit. J. Pharmacol. 142, 231-255.

- Hernández-Ledesma, B., Amigo, L., Recio, I., Bartolome, B., 2007. ACE-inhibitory and radical-scavenging activity of peptides derived from beta-lactoglobulin f(19-25). Interactions with ascorbic acid. J. Agric. Food Chem. 55, 3392-3397.

- Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., Amigo, L., 2005. Preparation of antioxidant enzymatic hydrolyzates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. J. Agric. Food Chem. 53, 588-593.

- Hernández-Ledesma, B., Hsieh, C. –C., de Lumen, B. O., 2009a. Lunasin, a novel seed peptide for cancer prevention. Peptides 30, 426-430.

- Hernández-Ledesma, B., Hsieh, C. –C., de Lumen, B. O., 2009b. Lunasin and Bowman-Birk protease inhibitor (BBI) in US commercial soy foods. Food Chem. 115, 574-580.

- Hernández-Ledesma, B., Hsieh, C. –C., de Lumen, B. O., 2009c. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. Biochem. Biophys. Res. Commun. 390, 803-808.

- Hernández-Ledesma, B., Hsieh, C. –C., de Lumen, B. O., 2013. Chemopreventive properties of peptide lunasin: a review. Protein Pept. Lett. 20, 424-432.

- Hsieh, C.-C.; Hernández-Ledesma, B.; Jeong, H. J., Park, J. H., Lumen, B. O., 2010. Complementary roles in cancer prevention: Protease inhibitor makes the cancer preventive peptide lunasin bioavailable. PLoS ONE 5, e8890.

- Huang, W. –Y., Majumder, K., Wu, J., 2010. Oxygen radical absorbance capacity of peptides from egg white protein ovotransferrin and their interaction with phytochemicals. Food Chem. 123, 635-641.

- Jeong, J. B., de Lumen, B. O., Jeong, H. J., 2010. Lunasin peptide purified from *Solanum nigrum* L. protects DNA from oxidative damage by suppressing the generation of hydroxyl radical via blocking fenton reaction. Cancer Lett. 293, 58-64.

519 - Kim, H. J., Lee, E. K., Parke, M. H., Ha, Y. M., Juang, K. J., Kim, M. –S., Kim, M. K., Yu,
520 B. P., Chung, H. Y., 2013. Ferulate protects the epithelial barrier by maintaining tight junction
521 protein expression and preventing apoptosis in tert-butyl hydroperoxide-induced Caco-2 cells.
522 Phytother. Res. 27, 362-367.
523 - Kitts, D., Weiler, K., 2003. Bioactive proteins and peptides from food sources. Applications
524 of bioprocesses used in isolation and recovery. Curr. Pharm. Design 9, 1309-1323.
525 - Lam, Y., Galvez, A., de Lumen, B. O., 2003. Lunasin (TM) suppresses E1A-mediated
526 transformation of mammalian cells but does not inhibit growth of immortalized and
527 established cancer cell lines. Nutr. Cancer 47, 88-94.
528 - Laparra, J. M., Alegría, A., Barberá, R., Farré, R., 2008. Antioxidant effect of casein
529 phosphopeptides compared with fruit beverages supplemented with skimmed milk against
530 H_2O_2-induced oxidative stress in Caco-2 cells. Food Res. Int. 41, 773-779.
531 - LeBel, C. P., Ishiropoulos, H., Bondy, S. C., 1992. Evaluation of the probe 2',7'-
532 dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress.
533 Chem. Res. Toxicol. 5, 227-231.
534 - Li, Y. –W., Li, B., 2013. Characterization of structure–antioxidant activity relationship of
535 peptides in free radical systems using QSAR models: Key sequence positions and their amino
536 acid properties. J. Theor. Biol. 318, 29-43.
537 - Li, J., Liu, Y., Fan, L., Ai, L., Shan, L., 2011. Antioxidant activities of polysaccharides from
538 the fruiting bodies of *Zizyphus Jujuba* cv. *Jinsixiaozao*. Carbohydr. Polym. 84, 390-394.
539 - Manna, C., Migliardi, V., Sannino, F., De Martino, A., Capasso, R., 2005. Protective effects
540 of synthetic hydroxytyrosol acetyl derivatives against oxidative stress in human cells. J.
541 Agric. Food Chem. 53, 9602-9607.
542 - Martindale, J. L., Holbrook, N. J., 2002. Cellular response to oxidative stress: Signaling for
543 suicide and survival. J. Cell Physiol. 192, 1-15.

- Meisel, H., 2004. Multifunctional peptides encrypted in milk proteins. BioFactors 21, 55-61.

- Owuor, E. D., Kong, A. N. T., 2002. Antioxidants and oxidants regulated signal transduction pathways. Biochem. Pharmacol. 64, 1547.

- Pavlick, K. P., Laroux, F. S., Fuseler, J., Wolf, R. E., Gray, L., Hoffman, J., Grisham, M. B., 2002. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. Free Rad. Biol. Med. 33, 311-322.

- Pietta, P., 2000. Flavonoids as antioxidants. J. Nat. Prod. 63, 1035-1042.

- Power, O., Jakeman P., FitzGerald, R. J., 2013. Antioxidative peptides: enzymatic production, *in vitro* and *in vivo* antioxidant activity and potential applications of milk-derived antioxidative peptides. Amino Acids 44, 797-820.

- Rezaie, A., Parker, R. D., Abdollahi, M., 2007. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? Dig. Dis. Sci. 52, 2015-2021.

- Royall, J. A., Ischiropoulos, H., 1993. Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluo- rescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch. Biochem. Biophys. 302, 348-355.

- Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., Zucco, F., 2005. The Caco-2 cell line as a model of the intestinal barrier: influence of cells and culture-related factors on Caco-2 cell functional characteristics. Cell Biol. Toxicol. 21, 1-26.

- Silveira, S. T., Martínez-Maqueda, D., Recio, I., Hernández-Ledesma, B., 2013. Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. Food Chem. 141, 1072-1077.

- Udilova, N., Jurek, D., Marian, B., Gille, L., Schulte-Hermann, R., Nohl, H., 2003. Induction of lipid peroxidation in biomembranes by dietary oil components. Food Chem. Toxicol. 41, 1481-1489.

- Wang, H., Joseph, J. A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic. Biol. Med. 27, 612-616.

- Wijeratne, S. S. K., Cuppett, S. L., 2007. Oxidative stress induced by lipid hydroperoxides in the intestine - A review. Agro Food Ind. Hi-Tech 18, 27-29.

- Xie, N., Wang, C., Ao, J., Li, B., 2013. Non-gastrointestinal-hydrolysis enhances bioavailability and antioxidant efficacy of casein as compared with its in vitro gastrointestinal digest. Food Res. Int. 51, 114-122.

- Yokomizo, A., Moriwaki, M., 2006. Effects of flavonoids on oxidative stress induced by hydrogen peroxide in human intestinal Caco-2 cells. Biosci. Biotechnol. Biochem. 70, 1317-1324.

- Zhang, Q. –X., Ling, Y. –F., Sun, Z., Zhang, L., Yu, H. –X., Mburu Kamau, S., Lu, R. –R., 2012. Protective effect of whey protein hydrolysates against hydrogen peroxide-induced oxidative stress on PC12 cells. Biotechnol. Lett. 34, 2001-2006.