Multifunctional Activity of Soybean Protein Hydrolysates: Focus on the Hypocholesterolemic and Anti-Diabetic Effects.

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Abstract

This study was aimed at evaluating the hypocholesterolemic and anti-diabetic effects of peptic (P) and tryptic (T) hydrolysates from soybean protein. Both hydrolysates inhibit the HMGCoAR activity increasing the LDLR on HepG2 cell membranes. Soybean P increases LDLR protein levels by 51.5±11.6% and 63.0±6.9% (0.5 – 1.0 mg/mL) vs. the control, whereas Soybean T by 55.2±9.7% and 85.8±21.5% (0.5 – 1.0 mg/mL) vs. the control. This improved the HepG2 capacity to uptake LDL from the extracellular environment with a final hypocholesterolemic effect. Moreover, Soybean P reduced *in vitro* the DPP-IV activity by 16.3±3.0% and 31.4±0.12% (1.0 and 2.5 mg/mL), vs. the control, whereas Soybean T by 15.3±11.0% and 11.0±0.30% (1.0 and 2.5 mg/mL) vs. the control. Finally, both Soybean P and Soybean T inhibit *in situ* the DPP-IV activity on human intestinal Caco-2 cells. This investigation may help explaining the activities observed in experimental and clinical studies.

Keywords: Bioactive peptides, Caco-2 cells, DPP-IV, Soybean hydrolysates, LC-MS/MS, LDL-receptor.

INTRODUCTION

In addition to the nutritional value, food proteins display health-promoting functions through their ability to modulate some intracellular pathways. Most of the physiological activities provided by food proteins depend on peptide sequences encrypted in the parental protein, which are delivered by digestion, absorbed intact by intestinal cells, and transported to the target organs where they exert their effects.[1] In particular, many food protein hydrolysates exert antimicrobial, immunomodulatory, anti-oxidative, hypocholesterolemic, hypotensive, and anti-diabetic activities.[2, 3] Owing to the presence of numerous bioactive peptides, these protein hydrolysates may provide more than one biological activity, eliciting therefore multiple health benefits. For this reason, the production of hydrolysates with a multifunctional behavior represents a valid strategy for the development of new generations of functional foods and nutraceuticals.[4]

Certainly, milk protein hydrolysates are one of the most investigated food sources of bioactive peptides. In fact, it has been demonstrated that they may provide beneficial effects on the cardiovascular, gastrointestinal, immune, and nervous systems,[5] whereas egg-yolk protein hydrolysates are endowed with antioxidant, angiotensin converting enzyme (ACE) inhibitory and anti-diabetic activities.[6] Also hydrolysates from plant proteins are attractive. Peptic and tryptic hempseed protein hydrolysates possess hypocholesterolemic and anti-diabetic activities that have been investigated from a molecular and functional point of view in human hepatic HepG2 and intestinal Caco-2 cells,[7-9] whereas lupin protein hydrolysates are hypocholesterolemic.[10-12]

Soybean represents another promising source of protein hydrolysates with a multifunctional behavior. Even though the composition varies with the variety and the location and climate of the growing, soybean is in average composed of ~35–40% protein, ~20% lipid, ~9% dietary fiber, and ~8.5% moisture.[13] Numerous clinical studies have associated soy food consumption with a reduced risk of developing some chronic diseases, such as obesity, hypercholesterolemia, and insulin-resistance/type II diabetes. [14] In particular, soy foods are useful to decrease total and low-density lipoprotein cholesterol (LDL-C) levels in the presence of high and mild hypercholesterolemia.[15-17] This activity

is linked to the modulation of LDL receptors (LDLR). Other experimental and clinical studies have suggested that soy food consumption is also beneficial for reducing plasma glucose levels with anti-diabetic effects. [18-20] As for the active substance in soy foods, the protein plays a role in cholesterolemia reduction[21, 22] and some hypocholesterolemic and anti-diabetic peptides have been already identified in the sequences of glycinin and β-conglycinin, two major soybean globulins.[23, 24, 25]

Until now, however, the mechanisms through which soybean proteins exert the hypocholesterolemic and anti-diabetic effects have not been elucidated yet in detail. In order to fill this gap, a total protein extract from soybean was hydrolyzed either with pepsin (Soybean P) or trypsin (Soybean T) and, after analysis by mass spectrometry (MS), the biological activity was evaluated either *in vitro* with biochemical assays or *in situ* using suitable cell models. Specifically, the cholesterol-lowering effects were investigated initially by measuring the direct inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) and then by evaluating the capacity to modulate the activity of the LDLR in the human hepatic HepG2 cell line, whereas the anti-diabetic effects were investigated by determining the inhibition of dipeptidyl peptidase-IV (DPP-IV) activity either *in vitro*, using the isolated enzyme, or *in situ* on human intestinal Caco-2 cells, where the DPP-IV enzyme is abundantly expressed on the apical cell side. [26]

MATERIAL & METHODS

Materials. The HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin/streptomycin, chemiluminescent reagent, and 96-well plates were purchased from Euroclone (Milan, Italy). Bovine serum albumin (BSA), the human HMGCoAR activity assay kit, Tris-HCl, ethylenediamine tetra-acetic acid (EDTA), acetonitrile (ACN), and NaCl were from Sigma-Aldrich (St. Louis, MO, USA). Janus green was bought from

Abcam (Cambridge, UK), while the antibodies against anti-rabbit Ig-HRP was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, US). Antibody against LDLR and the 3,3',5,5'-tetramethylbenzidine (TMB) substrate were obtained from Pierce (Rockford, IL, US). LDL-DyLight™ 550 and the DPP-IV assay kit were from Cayman Chemical Company (Ann Arbor, MI, US). Soybeans were purchased in a local supermarket.

Preparation of the soy protein isolate (SPI) and enzymatic hydrolysis. The total protein extract was obtained from soybeans as previously reported.[27] Briefly, proteins were extracted from 2 g of defatted flour dispersed in 100 mM Tris-HCl/0.5 M NaCl buffer (1:10 w/v), pH 8.2, for 2 h at 4 °C under magnetic stirring. The solid residue was eliminated by centrifugation at 6500 g, for 20 min at 4 °C, and the supernatant was dialyzed against 100 mM Tris-HCl buffer, pH 8.2, for 24 h at 4 °C. The protein content was assessed according to the method of Bradford, using bovine serum albumin (BSA) as standard. For the enzymatic hydrolysis, the soy protein extract was initially dissolved in Tris-HCl buffer 100 mM at pH 8. The tryptic hydrolysis was performed directly in the same buffer adding trypsin (4 mg/mL in 1 mM HCl) in a ratio of 1:50 (E/S) (w/w). After 16 h incubation, the digestion was stopped heating the solution for 5 minutes at 95 °C. The peptic hydrolysis, instead, was performed adjusting the pH to 2 by adding 1 M HCl to the total protein extract solution. The enzyme solution (4 mg/mL in NaCl 30 mM) was added in a ratio of 1:50 (E/S) (w/w). The mixture was incubated for 16 h and the enzyme inactivated changing the pH to 7 by adding 1 M NaOH. Both peptic and tryptic hydrolysates mixtures were purified by ultrafiltration through 3 kDa cut-off centrifuge filters (Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 12,000 g for 30 min at 4 °C.

Characterization of soy protein hydrolysates by mass spectrometry and data analysis. Both tryptic and peptic hydrolysates were purified using SepPak C18 cartridges (Thermo Fisher Scientific, Life Technology, Milan Italy), dried in a Speed-Vac (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and then reconstituted with 20 μL of a solution of 2% ACN containing

0.1% formic acid. Aliquots of 5 µL of tryptic and peptic hydrolysates were injected in a nano-chromatographic system HPLC-Chip (Agilent Technologies, Palo Alto, CA, USA). The analysis was conducted on a SL IT mass spectrometer; 5 µL of each sample was loaded onto a 40 nL enrichment column (Zorbax 300SB-C18, 5 µm pore size), and separated onto a 43 mm × 75 µm analytical column packed (Zorbax 300SB- C18, 5 µm pore size). Separation was carried out in gradient mode at a flowrate of 300 nL/min. The LC solvent A was 95% water, 5% ACN, 0.1% formic acid; solvent B was 5% water, 95% ACN, 0.1% formic acid. The nano pump gradient program was as follows: 5% solvent B (0 min), 50% solvent B (0–50 min), 95% solvent B (50–60 min), and back to 5% in 10 min. The drying gas temperature was 300 °C, flow rate 3 L/min (nitrogen). Data acquisition occurred in positive ionization mode. Capillary voltage was −1970 V, with endplate offset −500 V. Full scan mass spectra were acquired in the mass range from m/z 300 to 2000 Da. LC-MS/MS analysis was performed in data-dependent acquisition AutoMS(n) mode. In order to increase the number of identified peptides, three technical replicates (LC–MS/MS runs) were run for each hydrolysate. The MS/MS data were analyzed by Spectrum Mill Proteomics Workbench (Rev B.04.00, Agilent), consulting the *Glycine max* (251326 entries) protein sequences database downloaded from the National Center for Biotechnology Information (NCBI). The enzymes selected were pepsin and trypsin for the analysis of the peptic and tryptic hydrolysates, respectively. Two missed cleavages were allowed to each enzyme used; peptide mass tolerance was set to 1.2 Da and fragment mass tolerance to 0.9 Da. For quality assignment, a sequence tag length > 4 was used. Threshold used for protein identification score ≥ 10; Scored Peak Intensity (SPI) % ≥ 70%; autovalidation strategy both in peptide mode and in protein polishing mode was performed using FDR cut-off ≤ 1.2 %.

Determination of degree of hydrolysis (DH). The degree of hydrolysis was determined by OPA assay, following a literature procedure[7] with some modifications. Additional details are reported in Supporting information.

HMGCoAR activity assay. The evaluation of the *in vitro* activity of HMGCoAR (EC 1.1.1.88.) was performed following the manufactory instructions and a procedure previously described.[12] Briefly, 0.25, 1.0, and 2.5 mg/mL of tryptic hydrolysate or 1.0, and 2.5 mg/mL of peptic hydrolysate were incubated with the HMG-CoA reductase (catalytic domain) (2 µL). The absorbance variation at 340 nm was monitored by Synergy H1 (BioTek Germany, Bad Friedrichshall, Germany) at time 0 and 10 min. The HMGCoA-dependent oxidation of NADPH and the inhibition properties of soybean hydrolysate were measured by the absorbance reduction, which is directly proportional to the enzyme activity.

Cell culture conditions. The HepG2 and Caco-2 cell lines were cultured following procedures which had been previously optimized.[10]

Cell fixation and in cell Western (ICW). Treated HepG2 cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT) and samples were processed for ICW assay following a procedure previously optimized [28] and described in "Supporting Information".

Fluorescent LDL uptake cell-based assay. The fluorescent LDL uptake cell-based assay was carried out using a procedure previously reported.[29] Additional details are reported in Supporting information.

***In vitro* DPP-IV activity assay.** The *in vitro* experiments aimed at evaluating the ability of the peptic and tryptic hydrolysates (concentrations 1.0 and 2.5 mg/mL) to inhibit the DPP-IV (EC 3.4 .14.5) enzyme were performed using the procedure previously reported.[30] Additional details are reported in Supporting information.

***In situ* DPP-IV activity assay.** A total of 5×10^4/well Caco-2 cells were seeded in black 96-well plates with clear bottom and cells were treated with 1.0, 2.5, and 5.0 mg/mL of Soybean P and Soybean T hydrolysates for 24 h. An aliquot of 100.0 µL of Gly-Pro-AMC substrate at the concentration of 50.0 µM in PBS ($Ca^{2+/}Mg^{2+}$ free) was added in each well. Fluorescence signals (ex./em. 350/450 nm) were measured using a Synergy H1 instrument (Biotek, Bad Friedrichshall, Germany) after 5 min of incubation.

Statistically Analysis. Statistical analyses were carried out by One-way ANOVA followed by Dunnett's test and by t-student using Graphpad Prism 6 (Graphpad, La Jolla, CA, USA). Values were expressed as means ± SD; *P-values* < 0.05 were considered to be significant.

RESULTS

Preparation and analysis of peptic and tryptic hydrolysates. The peptic and tryptic hydrolysates were prepared as indicated in the "Materials and Methods" section and ultra-filtered with a 3 kDa cut-off. The DH of Soybean P and Soybean T, determined by the OPA method, were equal to 40.7%, and 46.5% respectively. Both hydrolysates were then characterized by HPLC-MS/MS. Figure 1A reports the total ion current (TIC) of the MS/MS of eluted peptides, while Table S1 and S2 (see supporting materials) report the identified peptides in Soybean P and Soybean T and the corresponding parent proteins. The degree of hydrolysis (DH) as well as the molecular masses, lengths, and sequences of the peptides generated by the enzymatic proteolysis are important factors for producing protein hydrolysates with specific biological activities. Table 1 reports some main chemical features of each hydrolysate. The peptide lengths of the two hydrolysates are similar, in total from 8 to 27 amino acid residues. Based on the hydrophobicity of each residue, the average hydrophobicity of both hydrolysates was calculated to be equal to 45.4%, and 38.5%, respectively.

Table 1. Main chemical features of SoybeanP and SoybeanT hydrolysates

Entry	DH (%)	MW range (Da)	Peptide length range	Hydrophobicity (%)
Soybean P	40.7	881-2308	8-22	45.4
Soybean T	46.5	1116-2755	9-27	38.5

Figure 1B shows the MW distribution profiles of each hydrolysate: they include peptides with MW < 1 kDa, 1–1.2 kDa, 1.2–1.5 kDa, 1.5–2 kDa, and > 2 kDa. The hydrolytic efficiency of the two enzymes are dissimilar: in fact, Soybean P is characterized by short and very short peptides, some of which with MW smaller than 1 kDa, whereas Soybean T contains large amounts of medium and long peptides. In particular, only the latter contains numerous peptides with a MW larger than 2 kDa. In conclusion, it appears that, at least in the applied conditions, the pepsin hydrolysis was more extensive than the trypsin one.

The peptides distribution as a function of the length and the hydrophobicity of each subgroup are shown in Figures 1C and 1D. Briefly, Soybean P hydrolysate contains 22.2% peptides with lengths ranging from 8 to10 amino acid residues and an average hydrophobicity of 48.1 kcal mol^{-1}, 73.6% peptides with 11-20 amino acid residues length and an average hydrophobicity of 44.5 kcal mol^{-1}, and 4.2% of peptides with a length of 20-21 amino acids and an average hydrophobicity of 50.7 kcal mol^{-1} (Figure 1C). On the contrary, Soybean T contains 6.2% peptides with a 9-10 amino acid residues length and an average hydrophobicity of 32.2 kcal mol^{-1}, 67.2% peptides with a length of 11-20 amino acid residues and an average hydrophobicity of 39.2 kcal mol^{-1}, and 26.6% peptides with a length of 20-27 amino acid and an average hydrophobicity of 40.4 kcal mol^{-1} (Figure 1D). In practice, in all subclasses, the average hydrophobicity of pepsin peptides was larger than that of trypsin peptides.

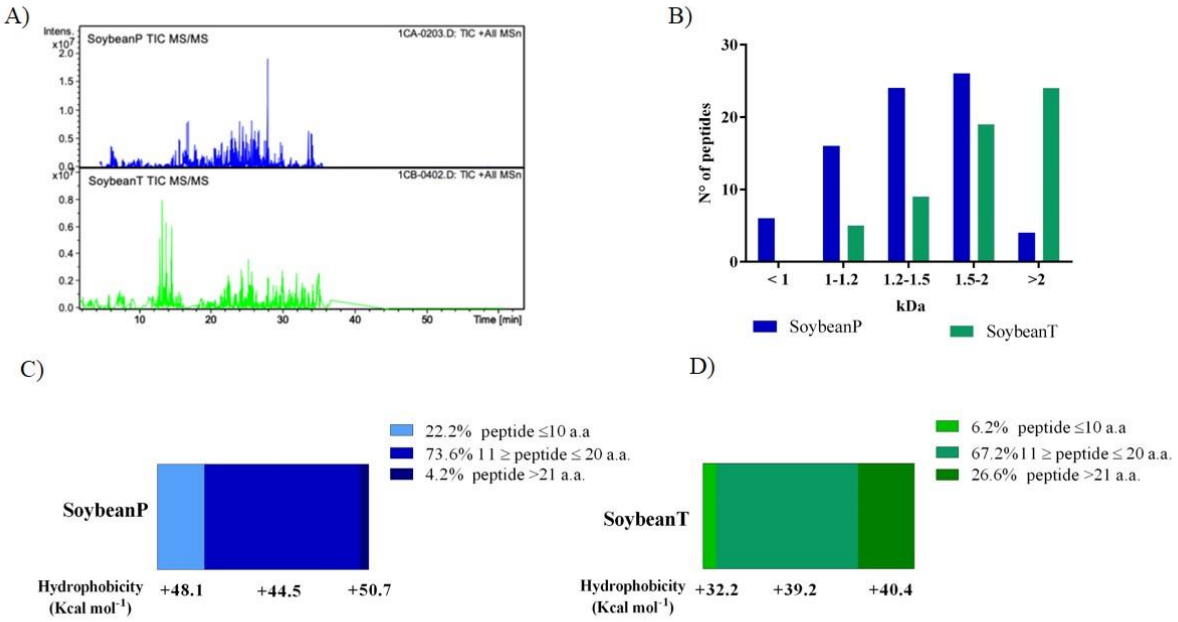

Figure 1. Characterization of the peptic and tryptic hydrolysates. A) Total ion current (TIC) of the MS/MS of Soybean P and Soybean T. B) Molecular weight distribution of peptides identified in Soybean P and Soybean T hydrolysates. C) Length and hydrophobicity distribution of the peptides identified in Soybean P. D) Length and hydrophobicity distribution of the peptides identified in Soybean T.

***In vitro* inhibition of the activity of HMGCoAR**. An *in vitro* assay was used to investigate the direct ability of Soybean P and Soybean T to inhibit the activity of HMGCoAR. The results are shown in **Figure 2.** Both hydrolysates dropped the HMGCoAR activity, but with very different efficacies. In fact, whereas Soybean P inhibited the enzyme with a statistical significance (-16.0±1.0%) only at the maximum dose (2.5 mg/mL) and was ineffective at 1.0 mg/mL, Soybean T induced a statistically significant reduction of the HMGCoAR activity at all tested concentrations, i.e. by 20.7±11.8% at 0.25 mg/mL, by 44.5±5.7% at 1.0 mg/mL, and by 76.9±4.9% at 2.5 mg/mL.

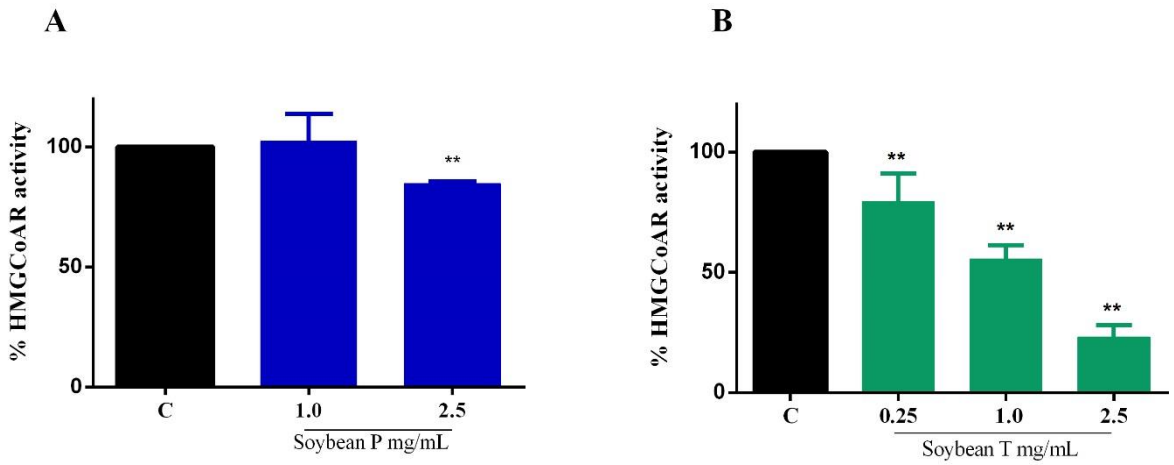

Figure 2. ***In vitro*** **inhibition of the HMGCoAR activity by Soybean P and Soybean T**. A) Percent activity of HMGCoAR after treatment with Soybean P hydrolysate. B) Percent activity of HMGCoAR after treatment with Soybean T. Bars represent the average ± sd of 3 independent experiments in duplicate. **p<0.01 versus untreated sample (C).

Modulation of the LDLR protein level on the HepG2 cell surfaces and of the ability of the HepG2 cells to uptake extracellular LDL-C. Based on the preceding biochemical results, the following experiments were aimed at assessing the capacity of Soybean P and Soybean T to modulate the LDLR localized on the HepG2 cell surface. This was done by using an in cell Western (ICW) assay recently developed by us.[28, 31] The results of this investigation are shown in **Figure 3A.** The treatments with both soybean hydrolysates produced an increase of the LDLR protein levels on the cell membrane: specifically, the treatment with Soybean P (0.5 – 1.0 mg/mL) produced an increase of the LDLR protein levels by 51.5±11.6% and 63.0±6.9%, respectively *vs.* the control sample, whereas the treatment with Soybean T (0.5 – 1.0 mg/mL) produced a rise by 55.2±9.7% and 85.8±21.5%, respectively, *vs.* the control sample.

Fluorescent LDL-C uptake experiments were performed for evaluating the cholesterol lowering properties of soybean peptides from a functional point of view. The fluorescent LDL uptake was examined in HepG2 cells following a 24 h incubation with Soybean P and Soybean T. The results of **Figure 3B** show that both hydrolysates increased the LDL-uptake in a statistically significant way

vs. the control. The treatment with Soybean P, at the concentration of 0.5 and 1.0 mg/mL, increased the LDL uptake by 48.2±16.5% and 68.6±6.5%, respectively, *vs.* the control, whereas Soybean T significantly raised the LDL-uptake by 46.6±6.2% and 87.8±14.0%, respectively, *vs*. the control at the concentration of 0.5, and 1.0 mg/mL.

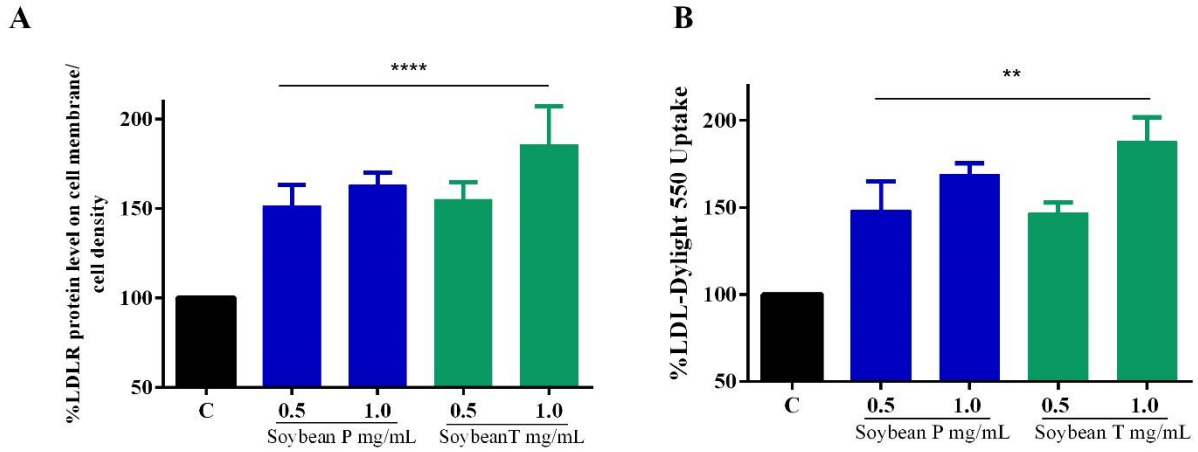

Figure 3. Effects of soy protein hydrolysates on LDLR protein levels and extracellular LDL-C uptake. A) Percent LDLR protein levels on HepG2 cell membranes after treatment with Soybean P and Soybean T. B) Percent uptake of LDL from the extracellular environment by HepG2 cells after treatment with Soybean P and Soybean T. Bars represent the average ± sd of 3 independent experiments in duplicate. ****p<0.0001, **p<0.01 versus untreated sample (C).

Inhibition of DPP-IV activity *in vitro* and *in situ* on human intestinal cells. The capability of both hydrolysates to inhibit the activity of DPP-IV was tested either *in vitro* or *in situ* on Caco-2 cells. **Figure 4A** shows the results of the experiments aimed at evaluating the inhibitory activity of the soybean hydrolysates against recombinant DPP-IV using fluorescent H-Gly-Pro-AMC as a substrate. The enzymatic reaction was monitored measuring the fluorescence signals, emitted at 465 nm, due to the free AMC group released after the cleavage of peptide H-Gly-Pro by DPP-IV. Each hydrolysate was screened at the final concentration of 1.0 and 2.5 mg/mL. Results indicate that Soybean P reduced the DPP-IV activity by 16.3±3.0% and 31.4±0.12% at 1.0 and 2.5 mg/mL, respectively, *vs* the control

sample, whereas Soybean T impaired the DPP-IV activity by 15.3±11.0% and 11.0±0.30% at 1.0 and 2.5 mg/mL, respectively, *vs* control samples (**Figure 4A**).

The ability of soybean protein hydrolysates to drop the DPP-IV activity expressed on the surface of the human intestinal cells was assessed by performing *in situ* experiments using non-differentiated Caco-2 cells (**Figure 4B**). In these experiments, Soybean P inhibited the DPP-IV activity by 15.4±6.4%, 19.6±7.1%, and 37.0±0.8% at 1.0, 2.5, and 5.0 mg/ml respectively, *vs* the control cells. Whereas, Soybean T reduced the DPP-IV activity by 17.0±8.7%, 25.2±0.7%, and 43.3±1.4% at 1.0, 2.5, and 5.0 mg/ml respectively, *vs* untreated cells (**Figure 4B**).

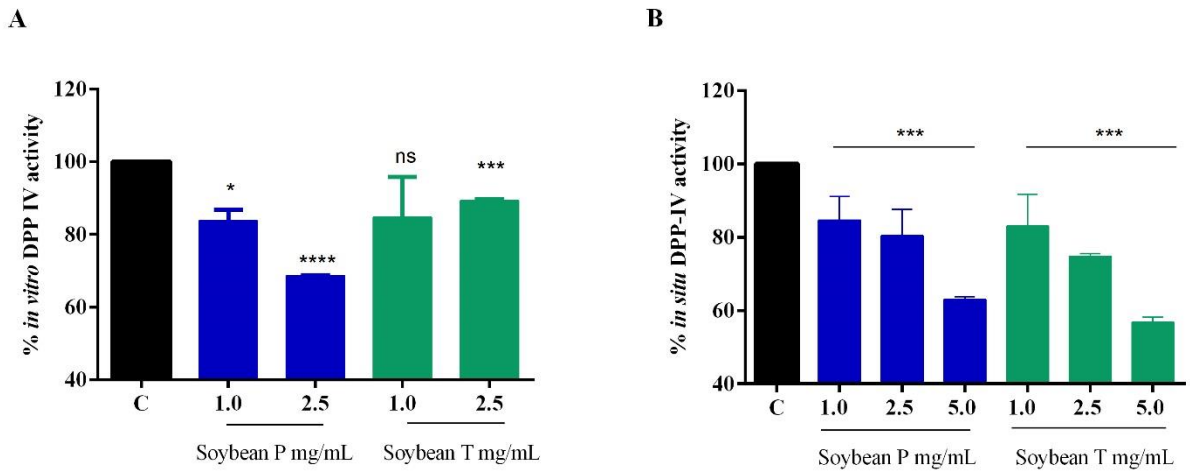

Figure 4. Effects of soy protein hydrolysates on DPP-IV activity. A) Percent *in vitro* activity of human recombinant DPP-IV after treatment with Soybean P and Soybean T. B) Percent *in situ* activity of DPP-IV expressed on Caco-2 cell membranes after treatment with Soybean P and Soybean T. Bars represent the average ± sd of 3 independent experiments in duplicate. ns: not significant, ****p<0.0001, ***p<0.001, *p<0.05 versus untreated sample (C).

DISCUSSION

A main limitation of available literature on bioactive peptides from food proteins is that most works rely exclusively on *in vitro* tests for assessing the biological activity: this is particularly true in the case of the inhibition of HMGCoAR and DPP-IV activity. A peculiarity of this paper, instead, is that the *in vitro* tests were implemented by cellular assays that permitted either to get a deeper insight in

the mechanism of action or, in parallel, to consider other relevant issues, such as metabolism (this is particularly true while employing Caco-2 cells). It is also important to underline that here only human enzymes and human cell lines were used in order to get a coherent picture of the phenomena.

Hypocholesterolemic activity of soybean protein hydrolysates. The first part of the work was aimed at assessing whether soybean protein hydrolysates exert a cholesterol-lowering effect and at elucidating the molecular mechanism of action. Being the rate-controlling enzyme of the cholesterol cellular biosynthetic pathway, HMGCoAR plays an important role in maintaining the intracellular cholesterol homeostasis. Its inhibition produces cholesterol-lowering effects and, for this reason, this enzyme is considered an important target for the development of new hypocholesterolemic agents.

The results of this work demonstrate that both soy protein hydrolysates impair the HMGCoAR activity *in vitro* but with different efficacies. In fact, Soybean T is about ten times more active than Soybean P, since the former drops the activity of HMGCoAR by 20.7% at the concentration of 0.25 mg/mL, whereas the latter by 16.0% at the concentration of 2.5 mg/mL (Figure 2). This difference is perfectly in line with the behavior of the tryptic and peptic hydrolysates from lupin protein investigated in a previous paper.[28] More in details, the former reduced *in vitro* the HMGCoAR activity by 37% at 0.25 mg/mL, while the latter by 17% at 2.5 mg/mL.[12] These differences underline the tight correlation between the physico-chemical properties and biological activities of these hydrolysates.

Considering that there is a good homology among the main storage proteins of the different legumes,[13, 32] it does not appear surprising that the peptic and tryptic protein hydrolysates from diverse species may display comparable bioactivities towards the same target. However, this is not true for all seeds: for example, the peptic and tryptic seed protein hydrolysates of hemp, belonging to the *Cannabaceae* family, inhibit the HMGCoAR activity in a comparable way, i.e. by 24.5 and by 24.6 respectively, at 0.25 mg/mL.[7, 9]

The evaluation of the *in vitro* activity, however, does not provide a comprehensive picture of the phenomenon. In fact, both soybean hydrolysates increase the ability of HepG2 cells to uptake the

297 LDL from the extracellular environment with a final cholesterol-lowering effect. In particular, after
298 treatment with Soybean P and Soybean T (0.5 mg/mL and 1.0 mg/mL), the extracellular LDL uptake
299 is increased by 48.2% and 68.6%, and by 46.6% and 87.8%, respectively. Apparently, in this case the
300 peptic hydrolysate is only slightly less active than the tryptic one. The improved ability to uptake
301 LDL cholesterol by HepG2 cells is linked to an growth of the LDLR protein levels localized on the
302 cellular membrane (Figure 3A-B). Similar results have been obtained also treating the same cells with
303 peptic and tryptic lupin protein hydrolysates: at the concentration of 1.0 mg/mL, Lupin P increases
304 the ability of HepG2 to uptake LDL by 42% and Lupin T by 70%, respectively.[12] From a molecular
305 point of view, the functional ability of hepatic cells to uptake the LDL from the extracellular
306 environment is correlated with an up-regulation of the LDLR protein levels. Also hempseed peptides
307 positively modulate the LDLR pathway inducing an increased LDL clearance by hepatic cells.[9]
308
309 **DPP-IV inhibitory activity of soybean peptides.** Our findings clearly point out that Soybean P and
310 T peptides drop the DPP-IV activity *in vitro* on the human recombinant enzyme and *in situ* on human
311 intestinal Caco-2 cells (Figure 4). Starting from the *in vitro* assays, Soybean P reduced the enzyme
312 activity by 16.3% and 31.4%, whereas Soybean T by 15.3% and 11.0%, respectively, at 1.0 and 2.5
313 mg/mL. These data are in line with recent studies demonstrating that the protein hydrolysates from
314 germinated and non-germinated soybean, obtained after simulated gastrointestinal digestion, show a
315 modest ability to inhibit the DPP-IV activity,[33, 34] as well as with the activity of other food-derived
316 protein hydrolysates.[35] For example, a hydrolysate of Atlantic salmon skin gelatin generated using
317 Flavorzyme® inhibits the activity of porcine DPP-IV by 45.0% at 5.0 mg/ml[36] and a hydrolysate of
318 Japanese rice bran protein, produced with Umanizyme G®, by 50% at 2.3 mg/ml.[37] Finally, the peptic
319 and tryptic hydrolysates of hempseed protein inhibit human DPP-IV activity by 32.0% and 17.5%,
320 respectively, at 1.0 mg/ml.[8]
321 Considering that the enzymatic hydrolysis of total protein extracts generates thousands peptides out
322 of which numerous may be active, it is certainly possible to hypothesize that the observed biological

effects depend on the combinatorial effects of numerous bioactive species. Certainly, the enzymatic hydrolysis is a very crucial step for the generation of bioactive protein hydrolysates. In this context, many variables should be taken into account, i.e. the hydrolytic enzyme, enzyme/substrate ratio, pH, and processing kinetics, which may all impact on the generated peptide sequences as well as on the concentration of the bioactive species. In light of these observations, it seems useful to compare the *in vitro* DPP-IV inhibitory activities of these hydrolysates with those of the hydrolysates obtained treating hempseed proteins with the same enzymes and in the same conditions. In both cases, the peptic hydrolysate is more active than the tryptic one, confirming the importance of a suitable selection of the hydrolytic enzyme [7].

It is useful to underline, that both studies were carried out on the human DPP-IV enzyme, whereas most published studies have been performed using porcine DPP-IV. Although the sequence of this enzyme is highly preserved among mammalian species, there is evidence that porcine and human DPP-IV differ in their susceptibility to the inhibition exerted by food-derived peptides.[38] Being generally stronger the inhibition on the porcine DPP-IV, the employment of this enzyme generates an overestimation of the inhibitory potency impairing the comparison among data obtained with different assays.[38]

Another specific feature of this work is the employment of an *in situ* intestinal cell-based assay for evaluating the inhibition of DPP-IV activity. This is a very important issue, because DPP-IV is abundantly expressed on the luminal surface of the enterocytes and therefore any potential inhibitor deriving from food digestion is likely to first interact with intestinal DPP-IV and other intestinal peptidases, before being absorbed. This certainly exposes the peptides to the risk of further metabolic degradation before being released into circulation, where they can interact with the soluble and vascular endothelial form of DPP-IV, thus affecting circulating gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) levels. Taking all these facts into account, this *in situ* intestinal cell-based assay is certainly much more advantageous than the traditional *in vitro* test. [8, 26] To confirm the importance of this issue, Soybean P is 2-times less active *in situ* on Caco-2 cells (37% inhibition

at 5.0 mg/mL) than *in vitro* on the recombinant human DPP-IV enzyme (31.4% inhibition at 2.5 mg/mL). In the same way, the incubation with Caco-2 cells impairs the inhibitory potency of peptic and tryptic hempseed hydrolysates, with a greater effect on the peptic one.[8] In addition, some soybean peptides able to inhibit DPP-IV activity[26, 39] are partially degraded after 2 h incubation with mature intestinal cells.[39] In fact, the intestinal brush border is a very complex physiological environment where a myriad of active proteases and peptidases are expressed that might metabolize food peptides modulating their bioactivity. Therefore, this organ acts not only as a major physiological barrier against the external environment permitting the absorption of valuable nutrients, but also actively participate to the modulation of the physico-chemical profiles of food protein hydrolysates, through the metabolic activity of its proteases.

Our results, however, indicate that the activities of Soybean P and Soybean T are influenced in a different way by the intestinal proteases, since only the activity of the former hydrolysate is greatly reduced in the Caco-2 assay. This outcome may possibly be explained with the diverse metabolic degradation also considering the peptide lengths distribution (Figure 1): Soybean P contains shorter peptides (22.2%, < 10 residues) than Soybean T (6.2%, < 10 residues), on the contrary, Soybean T contains longer peptides (26.6%, > 21 residues) than Soybean P (4.2%, > 21 residues). Possibly, the metabolic degradation of the shorter bioactive peptides of Soybean P may produce so short peptides that the activity is lost, whereas the metabolic degradation of the longer peptides in Soybean T may produce medium-length peptides with preserved or even enhanced activities.

In conclusion, to the best of our knowledge this is the first study reporting the cholesterol-lowering and anti-diabetic activities of the same soybean protein hydrolysates. Certainly, bioactive peptides derived from food proteins represent a very dynamic and challenging field, although many efforts are still necessary for a concrete exploitation in dietary supplements and functional foods. In this panorama, soybean protein hydrolysates are doubtlessly a promising source of peptides with multifunctional activities.

Abbreviations

ACE, angiotensin converting enzyme; ACN, acetonitrile, BSA, bovine serum albumin; DH, degree of hydrolysis; DMEM, Dulbecco's modified Eagle's medium; DPP-IV, dipeptidyl peptidase-IV; EDTA, ethylenediamine tetra-acetic acid; FBS, fetal bovine serum; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; ICW, in cell-western; LC, liquid chromatography; LDLR, low-density lipoprotein receptor; MS, mass spectrometry; MW, molecular weight; PBS, phosphate buffered saline; Soybean P, soybean hydrolyzed by pepsin; Soybean T, soybean hydrolyzed by trypsin; SPI, Scored Peak Intensity; TIC, total ion current; TMB, 3,3',5,5'-tetramethylbenzidine.

Supporting Information

The Supporting Information, which is available free of charge on the ACS Publications website at DOI: XXX, provides Table 1S and 2S reporting the identified soybean peptide lists and detailed information regarding the materials and methods section.

Author Contributions

C.L. conceived the project. C.L. designed and took care of the bioactivity characterization, while G.A. prepared soybean peptides, designed and performed experiments for the peptide identifications. C.L., G.A., and A.A wrote the manuscript. All authors critically reviewed the paper, and have approved the final article.

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401 ## Notes

402 The authors declare no competing financial interest.

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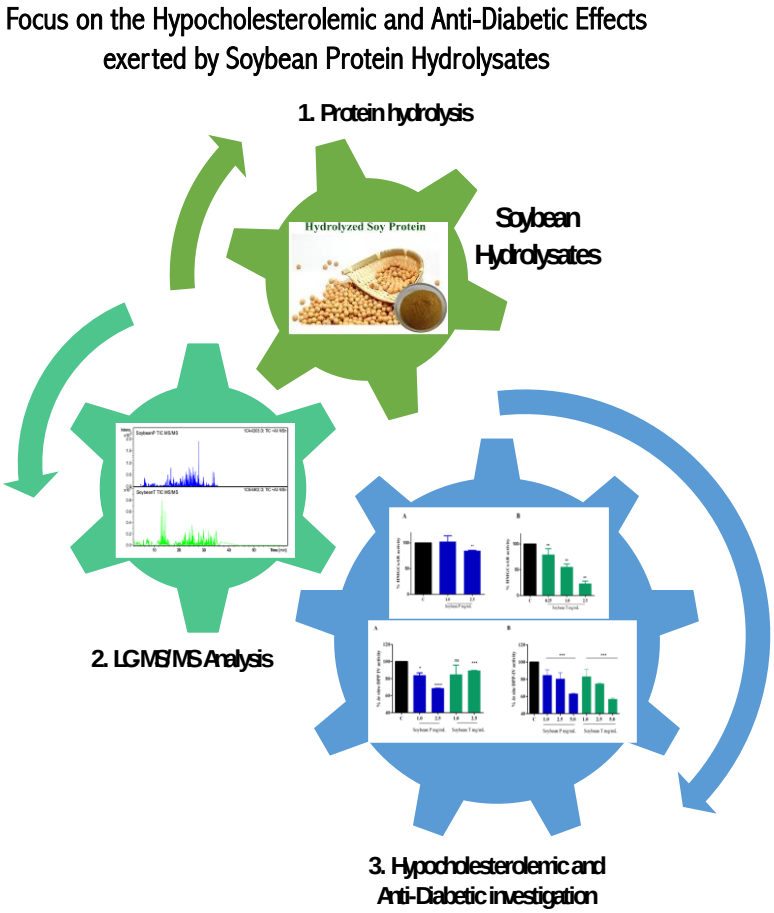

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512 TOC: experimental workflow for the characterization of the multifunctional behavior os soybean

513 protein hydrolysates.