

# Lupin Peptides Lower Low-Density Lipoprotein (LDL) Cholesterol through an Up-regulation of the LDL Receptor/Sterol Regulatory Element Binding Protein 2 (SREBP2) Pathway at HepG2 Cell Line

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## S Supporting Information

**ABSTRACT:** Previous experiments in suitable animal models and in mild hypercholesterolemic individuals have shown that the consumption of lupin proteins may be useful for controlling total and low-density lipoprotein (LDL) cholesterol levels. With the objective of providing evidence that peptides deriving from the hydrolysis of lupin proteins may be responsible of the observed activities and for investigating the mechanism of action, HepG2 cells were treated with lupin peptides obtained by either pepsin (P) or trypsin (T) hydrolysis, and molecular and functional investigations were performed on the LDL receptor/SREBP2 pathway. For the first time, this paper provides experimental evidence that lupin peptides are able to interfere with the HMGCoAR activity, up-regulating the LDL receptor (136 and 84% vs the control for P and T peptides, respectively, at 1 mg/mL) and SREBP2 proteins (148 and 73% vs the control for P and T peptides, respectively, at 1 mg/mL) via the activation of PI3K/Akt/GSK3 $\beta$  pathways and increasing the LDL uptake at HepG2 cell line (40 and 50% vs the control for P and T peptides, respectively, at 1 mg/mL). These results may be useful in explaining the activities observed in vivo in animals and humans treated with lupin protein.

**KEYWORDS:** *LDL receptor, LDL uptake, Lupinus albus, plant protein, functional foods*

## INTRODUCTION

Lupin is a protein-rich grain legume, which has been domesticated for a long time and cultivated on different continents, for either animal or human nutrition. This generic term indicates four species: *Lupinus albus* (white lupin), *Lupinus angustifolius* (narrow-leaf lupin), *Lupinus luteus* (yellow lupin), and *Lupinus mutabilis* (Andean lupin). The seeds of these plants have some favorable features; in particular, the protein percentage is comparable to that of soybean,<sup>1</sup> and the content of indispensable amino acids is only slightly inferior. Lupin is also a good source of minerals,<sup>1</sup> unsaturated fatty acids,<sup>2</sup> vitamins,<sup>1</sup> and tocopherols,<sup>3</sup> whereas the concentrations of isoflavones<sup>4</sup> and other antinutrients are low.<sup>5</sup> Experiments in rats have shown that the nutritional quality of *L. albus* protein is satisfactory.<sup>6</sup> Old species contained undesirable quinolizidine alkaloids that in modern cultivars were decreased to very low amounts,<sup>7</sup> permitting the direct use of these seeds for different applications.<sup>8,9</sup>

Besides these important nutritional features, lupin seed may also provide some health benefits, particularly in the area of hypertension<sup>10</sup> and dyslipidemia prevention. Some investigations in a rat model of hypercholesterolemia have demonstrated that diets containing either *L. albus* protein<sup>11,12</sup> or *L. angustifolius* protein<sup>13,14</sup> may significantly reduce both total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels versus control diets containing casein. The cholesterol-lowering activity of *L. albus* has also been confirmed in a hamster model of dyslipidemia.<sup>15</sup> Moreover, in a rabbit model of atheromatous plaque, *L. albus* protein has slowed plaque formation induced by a hyperlipidemic diet.<sup>16</sup> Finally, an uncontrolled clinical trial on

*L. albus*<sup>17</sup> and two controlled on *L. angustifolius*<sup>18,19</sup> have confirmed the hypocholesterolemic activity in humans. Despite these positive results, however, only very scarce data are available either on the mechanism of action or on the actual bioactive component(s) in lupin.

The favorable effects of plant proteins in cardiovascular prevention is well established; the first study on the hypocholesterolemic activity of soybean dates back to the 1970s.<sup>20</sup> Some relevant reviews and meta-analyses<sup>21–23</sup> summarize the numerous trials performed in the following years. In 1999 the U.S. Food and Drug Administration approved the health claim on the role of soybean protein in reducing the risk of cardiovascular disease.<sup>24</sup> The literature on soy may thus provide mechanistic information that may be also relevant in the case of other plant proteins, such as lupin. Studies in animal models and humans have demonstrated that the hypocholesterolemic effects of soy are due to the activation of LDL receptors (LDLR) that are relevant in the metabolic degradation of LDL-C. Soy protein was able to reverse the dramatic down-regulation of liver LDLR observed in rats on cholesterol/cholic acid dietary regimens versus casein,<sup>25</sup> and the expression of LDLR was increased in patients affected by familial hypercholesterolemia<sup>26</sup> and in moderately hypercholesterolemic individuals.<sup>27</sup> In parallel, in vitro and in vivo experiments have demonstrated that some

**Received:** February 19, 2014

**Revised:** June 19, 2014

**Accepted:** June 27, 2014

specific soy peptides are responsible for the LDLR modulation.<sup>28–32</sup> Nevertheless, the mechanism at the molecular level has not been investigated in detail yet. These bioactive peptides, initially encrypted in the soy protein sequences, are probably enzymatically released during digestion and absorbed.<sup>33</sup>

The majority of plasma cholesterol is transported by the LDL fraction, and the cellular uptake of LDL is mediated by the LDLR. The circulating level of LDL is determined in large part by its rate of uptake through the hepatic LDLR pathway.<sup>34,35</sup> Moreover, the LDLR plays a crucial role in Apo B turnover, being involved in determining the post-translation fate of apoB by increasing presecretory apoB degradation and mediating re-uptake of nascent lipoprotein particles.<sup>36</sup> In general, LDLR expression is finely tuned by changes in intracellular cholesterol.<sup>37</sup> A transcription factor known as the sterol-responsive element binding protein 2 (SREBP2) plays a critical role in LDLR mRNA expression.<sup>38,39</sup> Among SREBP2 gene targets, the 3-hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase (HMGCoAR) is particularly important. This enzyme plays a key role in the intracellular cholesterol biosynthesis, because it is the rate-controlling enzyme in the mevalonate pathway. After synthesis, SREBP2 forms a complex with the SREBP cleavage-activating protein (SCAP) and is localized in the endoplasmic reticulum (ER) as an inactive precursor (pro-SREBP2). Sterol deficiency results in the release of SREBP2/SCAP complex from ER and transport to the Golgi, where pro-SREBP2 is processed further, allowing the N-terminal fragment (mature SREBP2) to enter the nucleus and up-regulate transcription of LDLR and HMGCoAR.<sup>40</sup> In addition to SREBP2, other transcription factors may be involved in a context-dependent fashion in regulating the LDLR expression.

Recent studies<sup>41,42</sup> identify a crucial signaling pathway, via phosphatidylinositol-3-kinase (PI3K)/Akt, as an important player in the regulation of cellular lipid metabolism. This pathway is the best known for its role in promoting cell growth, proliferation, and survival through increased glucose utilization and prevention of apoptosis.<sup>43,44</sup> In particular, Akt has recently been implicated in a novel form of lipid metabolism regulation, through the SREBPs.<sup>45</sup> The physiological coordination between Akt and SREBPs pathways is needed to produce the lipids for new membrane synthesis, which in turn is required for growing and proliferating cells.<sup>41,42</sup> Even though many studies have been focused on the SREBP-1c isoform,<sup>45</sup> new efforts are directed to explore and to investigate the link between Akt and SREBP2.<sup>41,46</sup>

The objective of the present study was to characterize in detail the molecular mechanism at the basis of the cholesterol-lowering properties of *L. albus* protein observed in experimental and clinical investigations. On the basis of the hypothesis that the activity may depend on specific peptides encrypted in the protein sequences, a total protein extract from lupin seed was hydrolyzed either with pepsin (P peptides) or with trypsin (T peptides). The HepG2 cell line was treated with both kinds of peptides, and molecular and functional investigations were performed on the LDLR-SREBP2 and PI3K/Akt pathways, using a combination of techniques.

## MATERIALS AND METHODS

**Chemicals.** 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), RIPA buffer, HMGCoAR Assay Kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and wortmannin were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against HMGCoAR

were bought from Abcam (Cambridge, UK). Antibodies against  $\beta$ -actin, SREBP-2, rabbit Ig-HRP, and mouse Ig-HRP, PMSF, sodium orthovanadate inhibitors, and goat anti-rabbit Ig-HRP were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against the LDL receptor were from Pierce (Rockford, IL, USA). The inhibitor cocktail Complete Midi was from Roche (Basel, Switzerland). Mini protean TGX precast gel 7.5% and Mini nitrocellulose Transfer Packs were purchased from Bio-Rad (Hercules, CA, USA). LDL-DyLight 549 was from Cayman Chemical (Ann Arbor, MI, USA).

**Preparation and Analysis of the Pepsin and Trypsin Peptide Mixtures.** Lupin seeds of the species *L. albus* (cultivar Ares) were provided by Terrena (Matrignè-Ferchaud, France). The total protein extracts were obtained as previously reported.<sup>47</sup> Briefly, proteins were extracted from defatted flour with 100 mM Tris-HCl/0.5 M NaCl buffer, pH 8.2, for 2 h at 4 °C. The solid residue was eliminated by centrifugation at 6500g, 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 BSA as standard.

For the hydrolysis, the total protein extract was initially dissolved in Tris-HCl buffer 100 mM at pH 8, and then the pH was adjusted to the optimal hydrolysis conditions for each enzyme (pH 2 for pepsin and pH 8 for trypsin) by adding 1 M NaOH or 1 M HCl. The mixtures were incubated for 18 h, then the enzymes were inactivated, and the mixtures were purified by ultrafiltration through 3000 Da cutoff centrifuge filters (Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 12000g for 30 min at 4 °C. The peptide concentration in the permeates was measured according to a literature method,<sup>48</sup> based on chelating the peptide bonds by Cu(II) in alkaline media and monitoring the change of absorbance at 330 nm. Details are reported in a previous paper.<sup>47</sup>

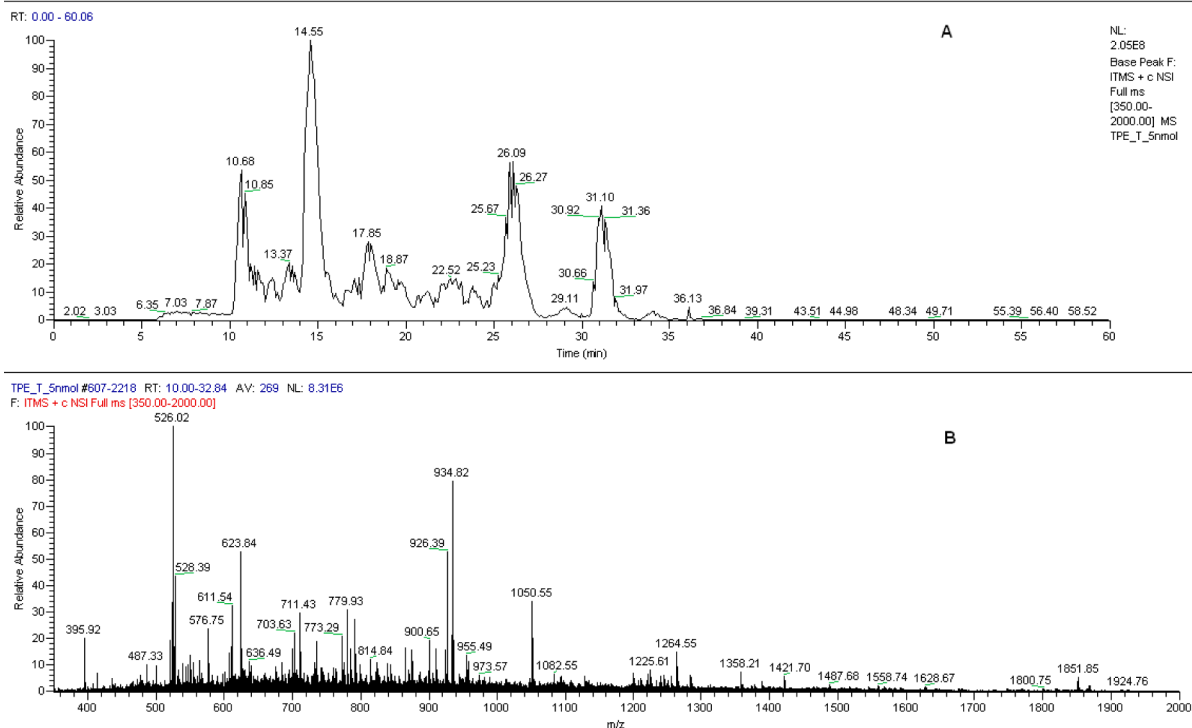
The hydrolyzed mixtures were acidified with formic acid to a final 10% concentration. Eight microliters of tryptic digest for each band was injected in a nanochromatographic system, UltiMate 3000 RSLC nano-System (Thermo Scientific). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 100  $\mu$ m i.d.  $\times$  2 cm, Thermo Scientific) for the cleanup and preconcentration. After cleanup, the valve was switched to place the trap column in series with a fused silica reverse-phase column (picoFrit column, C18, 2.7  $\mu$ m, New Objective). The peptides were eluted with a 30 min gradient from 4% buffer A (2% acetonitrile and 0.1% formic acid in water) to 60% buffer B (2% water and 0.1% formic acid in acetonitrile) at a constant flow rate of 300 nL/min. The chromatographic column was directly connected to an LTQ-XL mass spectrometer (Thermo Scientific) equipped with a nano spray ion source. Full scan mass spectra were acquired in the mass range from  $m/z$  350 to 2000 Da, and the five most intense ions were automatically selected and fragmented in the ion trap. Target ions already selected for mass spectrometry (MS/MS) were dynamically excluded for 30 s. The MS data were analyzed separately by Mascot search engine (version 2.3.01) using Proteome Discover software (v. 1.2.0 Thermo) and consulting Uniprot\_viridiplantae (30,264 sequences, 184,678,199 residues). Oxidation of methionine residues was set as variable modifications; two missed cleavages were allowed to trypsin; peptide mass tolerance was set to 1 Da and fragment mass tolerance to 0.8 Da; and an ion source cutoff of 20 was chosen. The false discovery rate obtained by Proteome Discover, consulting the Mascot decoy database, was <0.01. Tables 1S and 2S in the Supporting Information list the proteins and the unmatched peptides, with all relevant peptide signals, peptide sequences, ion score, variable modifications and mass errors.

**Cell Culture Conditions.** HepG2 cell line was bought from ATCC (HB-8065, ATCC from LGC Standards, Milan, Italy). The HepG2 cell line was cultured in DMEM high-glucose with stable L-glutamine supplemented with 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin (complete growth medium) and incubated at 37 °C under 5% CO<sub>2</sub> atmosphere. HepG2 cells were used for no more than 20 passages after thawing because the increase of the number of passages may change the cell characteristics and impair assay results.

**MTT.** HepG2 cells ( $3 \times 10^4$ /well) were seeded in 96-well plates and treated with 0.3, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/mL of P and T peptides, respectively, or vehicle (100 mM Tris) in complete growth media for 48 h. Subsequently, the treatment solvent was aspirated and 100  $\mu$ L/well of MTT

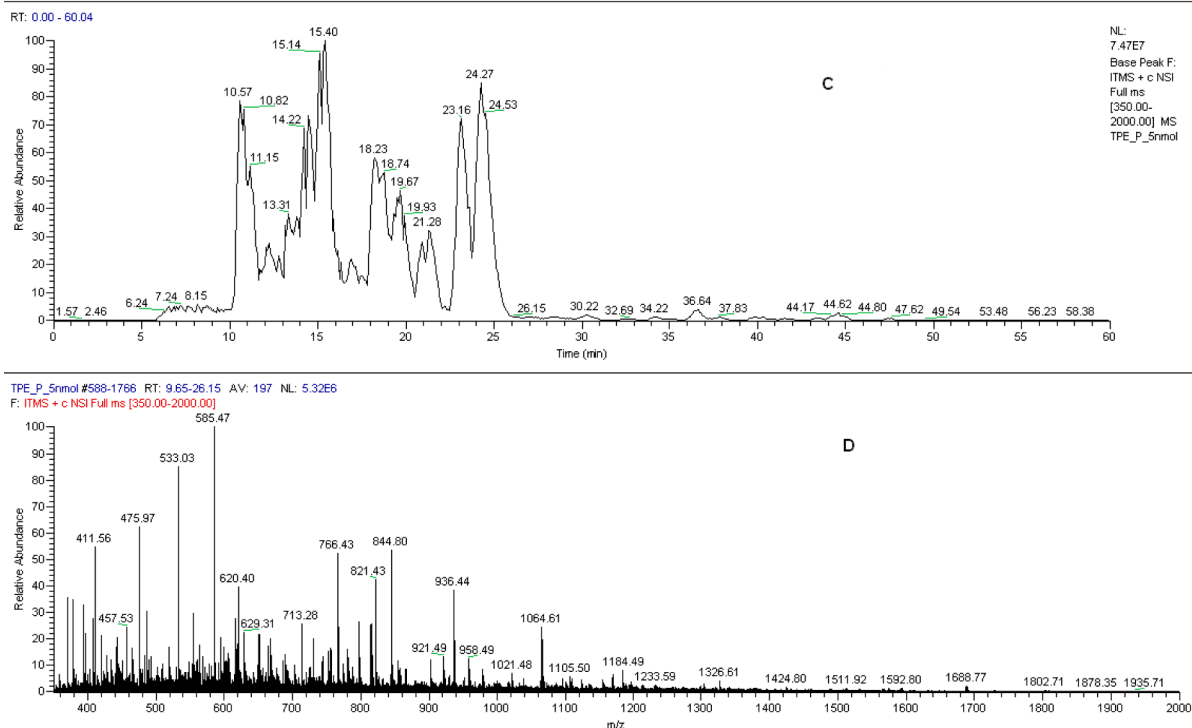
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**Figure 1.** Nano-LC MS/MS of lupin peptide mixtures: (A, B) chromatogram and full scan mass spectrum of T peptides; (C, D) chromatogram and full scan mass spectrum of P peptides.

filtered solution added for 48 h. After the incubation time, 0.5 mg/mL MTT solution was aspirated and 100  $\mu$ L/well of MTT lysis buffer (8 mM HCl + 0.5% NP-40 in DMSO) added. After 5 min of slow shaking, the absorbance at 575 nm was read on an LT 4000 spectrophotometer.

**HMGCoAR Activity Assay.** The assay buffer, the NADPH, the substrate solution, and the HMG-CoA reductase were provided in the

HMGCoAR Assay Kit (Sigma). The experiments were carried out following the manufacturer's instructions at 37 °C. In particular, each reaction (200  $\mu$ L) was prepared by adding the reagents in the following order: 1 $\times$  assay buffer; 0.1, 0.25, 0.5, 1.0, and 2.5 mg/mL of T peptides or 0.5, 1.0, and 2.5 mg/mL of P peptides or vehicle (C); NADPH (4  $\mu$ L); substrate solution (12  $\mu$ L); and finally HMG-CoA reductase

(catalytic domain) (2  $\mu$ L). Subsequently, the samples were mixed and the absorbance at 340 nm read by a Synergy H1 microplate reader from Biotek at times 0 and 10 min. The HMGC-CoA-dependent oxidation of NADPH and the inhibition properties of lupin peptides were measured by the absorbance reduction, which is directly proportional to the enzyme activity.

**Western Blot Analysis.** HepG2 cells ( $1.5 \times 10^5$ /well, 24-well plate) were treated with 0.5, 1, and 2.5 mg/mL of P and T peptides for 24 h. For the experiments in the presence of wortmannin, cells were treated with 1 mg/mL P and 0.5 mg/mL T peptides in the presence or absence of 1  $\mu$ M inhibitor for 24 h. After each treatment, cells were scraped in 40  $\mu$ L of ice-cold lysis buffer (RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100 sodium orthovanadate) and transferred in an ice-cold microcentrifuge tube. After centrifugation at 13300 rpm for 15 min at 4  $^{\circ}$ C, the supernatant was recovered and transferred into a new ice-cold tube. Total proteins were quantified according to the Bradford method, and 50  $\mu$ g of total proteins was loaded on a precast 7.5% sodium dodecyl sulfate–polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was pre-equilibrated with 0.04% SDS in H<sub>2</sub>O for 15 min at room temperature and transferred to a nitrocellulose membrane (Mini nitrocellulose Transfer Packs,) using a Trans-blot Turbo at 1.3 A and 25 V for 7 min. Target proteins, on milk-blocked membrane, were detected by primary antibodies as follows: rabbit anti-SREBP2, rabbit anti-LDLR, anti-HMGCAR, antipospho-Akt (ser473), antipospho-GSK3 $\alpha$ / $\beta$  (ser21/ser9), and anti- $\beta$ -actin. Secondary antibodies conjugated with HRP and a chemiluminescent reagent were used to visualize target proteins, and their signal was quantified using ImageJ software. The internal control,  $\beta$ -actin, was used to normalize loading variations. In the case of SREBP2, the band at 70 kDa, corresponding to the N-terminal fragment (mature SREBP2 protein), was detected and quantified.

**Fluorescent LDL Uptake Cell-Based Assay.** HepG2 cells ( $3 \times 10^4$ /well) were seeded in 96-well plates and kept in complete growth medium for 2 days before treatment. The third day, cells were treated with 1.0 mg/mL P peptides and 0.25 mg/mL T peptides, respectively, or vehicle (100 mM Tris) for 24 h. For the experiments in the presence of the PI3K inhibitor, HepG2 cells were treated with 1.0 mg/mL P peptides and 0.5 mg/mL T peptides in the presence or absence of 100 nM wortmannin and in the presence of the only inhibitor, as control, in DMEM without FBS for 24 h. At the end of the treatment periods, the culture medium was replaced with 75  $\mu$ L/well LDL-DyLight 549 working solution. The cells were additionally incubated for 2 h at 37  $^{\circ}$ C, and then the culture medium was aspirated and replaced with PBS 100  $\mu$ L/well. The degree of LDL uptake was measured using the Synergy H1 fluorescent plate reader from Biotek (excitation and emission wavelengths 540 and 570 nm, respectively).

**Statistical Analysis.** Statistical analyses were carried out by one-way ANOVA (Graphpad Prism 6) followed by Dunnett's test. Values were expressed as means  $\pm$  SEM; *P* values <0.05 were considered to be significant.

## RESULTS

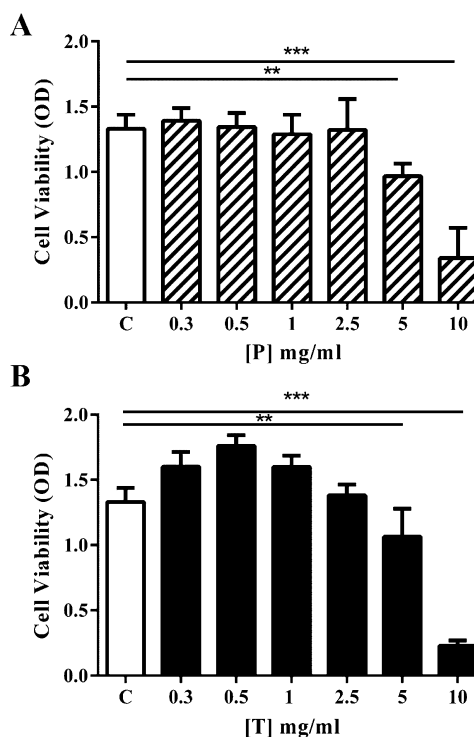
**Preparation and Analysis of the Peptide Mixtures.** A total protein extract from lupin seed was hydrolyzed with pepsin and trypsin to produce P peptides and T peptides, respectively. The two samples were analyzed by nano-LC-MS/MS. The chromatograms (Figure 1) showed two diverse profiles due to the different peptide compositions of the digested samples. The number of potentially bioactive peptides is very high in both samples, because more than 2000 peptides were detected in the pepsin-digested mixture and about 3000 in the trypsin-digested one (Tables 1S and 2S in the Supporting Information).

It was possible to assign only a small number of peptides to known lupin proteins by Mascot software consulting the Uniprot\_viridiplantae database. The other peptides were sequenced, but not assigned to protein hits, possibly due to the very incomplete sequencing of lupin proteins. The recognized

peptides in the P sample belong to the main storage proteins in lupin seed: 21 peptides to the vicilins [*L. albus* vicilin-like protein (Q53HY0) and *L. albus*  $\beta$ -conglutin (Q6EBC1)]; 7 peptides to the legumins [*L. angustifolius* conglutin- $\alpha$  3 (F5B8V8)], and 8 peptides to  $\gamma$ -conglutin [*L. albus* conglutin- $\gamma$  (Q9FSH9)], a sulfur-rich basic protein typical of lupin (4–5%).

The identified peptides in the T sample are the following: 16 peptides belong to the vicilins [*L. albus* vicilin-like protein (Q53HY0), *L. albus*  $\beta$ -conglutin precursor (Q6EBC1), and *L. angustifolius* conglutin  $\beta$  (B0YJF8)]; 4 to the legumins [*L. angustifolius* legumin-like protein (Q53I55)], and 2 to  $\delta$ -conglutin, a 2S protein [*L. albus* conglutin  $\delta$  seed storage protein precursor (Q99235)]. Two peptides were assigned to other plant proteins, probably because the lupin sequences are not present in the database: *Zea mays* actin partial (ADF3).

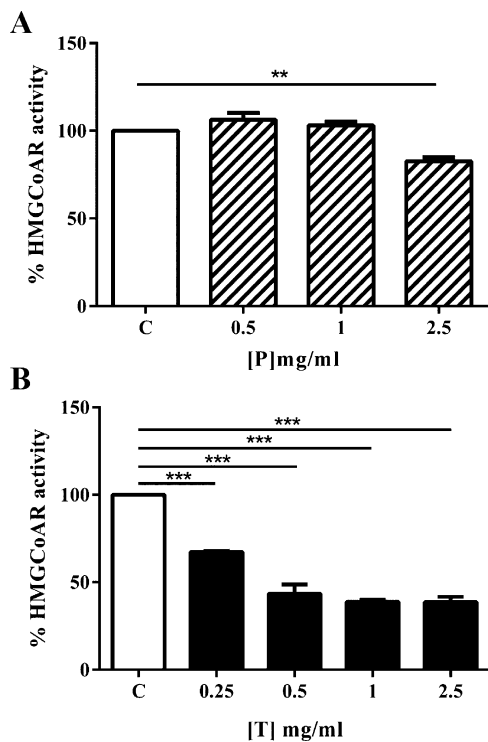
**HepG2 Cell Viability.** MTT experiments were performed to exclude the peptide doses with potential toxic effects on the HepG2 cell line. No significant cell mortality was observed up to 2.5 mg/mL after a 48 h treatment versus vehicle (C, 100 mM Tris), suggesting that neither P nor T peptides induce cell mortality in this dose range, whereas about 20 and 80% cell mortalities were observed at 5.0 and 10 mg/mL, respectively (Figure 2). For this reason, all of the following experiments,



**Figure 2.** HepG2 cell viability after P and T peptide treatment. Bar graphs indicate the results of MTT cell viability assay of HepG2 cells after P and T peptide treatment for 48 h. Data points represent averages  $\pm$  SEM of three independent experiments in triplicate. (\*\*) *P* < 0.001 and (\*\*\*) *P* < 0.0001 versus C. P, pepsin peptides; T, trypsin peptides; C, control.

aimed at investigating the molecular and functional effects of P and T peptides, were conducted using doses of  $\leq 2.5$  mg/mL.

**Effects of P and T Peptides on the Catalytic Domain of HMGCAR.** To check the direct ability of P and T peptides to inhibit HMGCAR, an in vitro assay was performed using the purified catalytic domain of this enzyme. Both P and T peptides



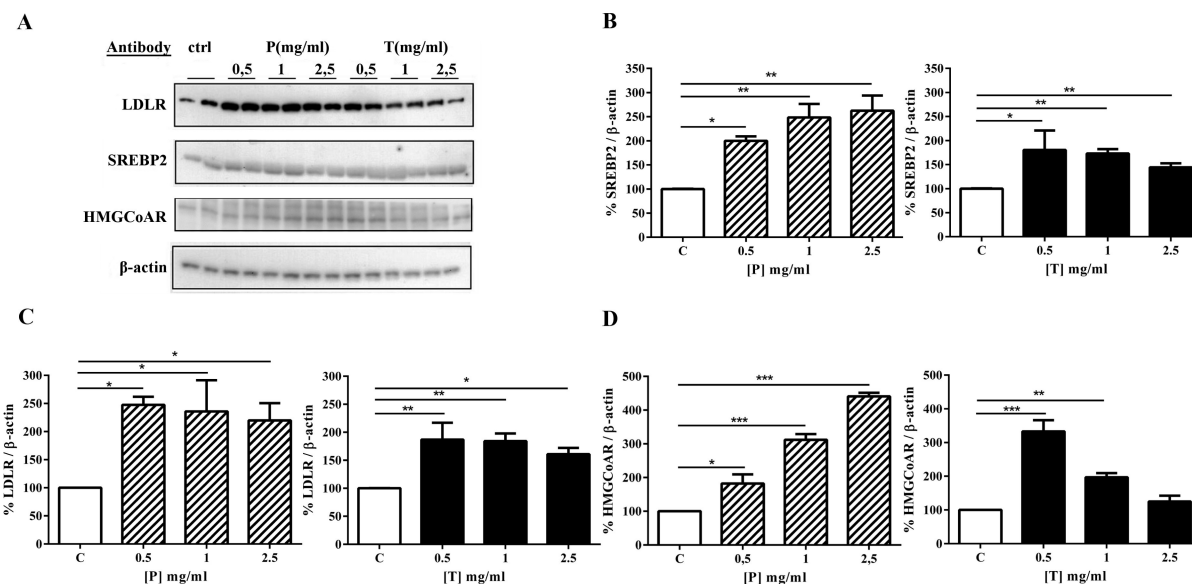
**Figure 3.** Lupin peptide effect on the catalytic domain of HMGCAR. Bars indicate the effects of P peptides (0.5, 1.0, and 2.5 mg/mL) (A) and T peptides (0.25, 0.5, 1.0, and 2.5 mg/mL) (B) on HMGCAR activity. HMGCAR, physiologically, catalyzes the four-electron reduction of HMG-CoA to coenzyme A (CoA) and mevalonate (HMG-CoA + 2NADPH + 2H<sup>+</sup> > mevalonate + 2NADP<sup>+</sup> + CoA-SH). In this assay, the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMGCAR in the presence of the substrate HMG-CoA, was measured spectrophotometrically. Data points represent averages  $\pm$  SEM of three independent experiments in triplicate. (\*\*\*)  $P < 0.001$  and (\*\*\*\*)  $P < 0.0001$  versus C. P, pepsin peptides; T, trypsin peptides; C, control.

were capable of inhibiting HMGCAR, but with very different activities and potencies (Figure 3). In fact, P peptides inhibited the enzyme with a statistical significance ( $-17\%$ ) only at the maximum tested dose (2.5 mg/mL), whereas they are ineffective at 0.5 and 1.0 mg/mL. On the contrary, T peptides showed a statistically significant reduction of the HMGCAR activity at all tested doses: in fact, the enzyme activity was reduced by 37% at 0.25 mg/mL, by 57% at 0.5 mg/mL, and by 61% at 1.0 and 2.5 mg/mL.

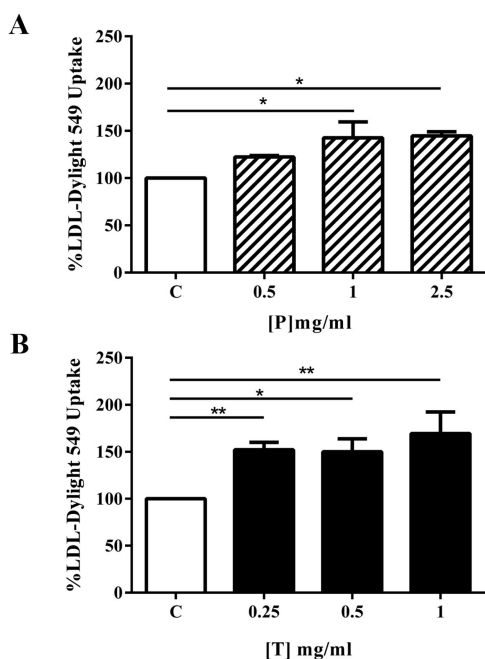
**Lupin Peptides Mediate the Up-regulation of LDLR-SREBP2 at HepG2 Cells.** HepG2 cells were treated for 24 h with P and T peptides at concentrations of 0.5, 1.0, and 2.5 mg/mL. Immunoblotting experiments showed that the treatments with lupin peptides induce an up-regulation of the SREBP2 protein level. In particular, P peptides up-regulate the SREBP2 protein level by 100, 148, and 162% versus the control, whereas T peptides increase the SREBP2 protein level by 80, 73, and 44% versus the control, after 0.5, 1.0, and 2.5 mg/mL treatments, respectively (Figure 4B).

In the same experiments, also the LDLR and HMGCAR protein levels were measured by immunoblotting. Figure 4C shows that both P and T peptides are able to induce a statistically significant up-regulation of LDLR protein in HepG2 cells versus the control, but with different behaviors. In particular, at 0.5, 1.0, and 2.5 mg/mL P peptides mediate 147, 136, and 120% inductions of the LDLR protein, whereas T peptides mediate a  $\sim 85\%$  up-regulation at 0.5 and 1.0 mg/mL and a 61% up-regulation at 2.5 mg/mL versus the control (Figure 4C). These results are in agreement with the up-regulation of SREBP2 protein level.

Both P and T peptides affect the HMGCAR levels (Figure 4D), but with very different behaviors and activities. Precisely, P peptides increase the synthesis of HMGCAR by 82% at 0.5 mg/mL, by 212% at 1.0 mg/mL, and by 340% at 2.5 mg/mL versus the control, that is, in a dose-dependent manner. On the contrary, treatment with T peptides enhances the production of HMGCAR by 233% at 0.5 mg/mL and by only 97% at 1.0 mg/mL, whereas it is practically inactive at 2.5 mg/mL.



**Figure 4.** Effect of P and T peptides on the SREBP2, LDLR, and HMGCAR protein levels. HepG2 cells ( $1.5 \times 10^5$ ) were treated with 0.5, 1.0, and 2.5 mg/mL of P and T peptides for 24 h, respectively. SREBP2, LDLR, HMGCAR, and  $\beta$ -actin immunoblotting signals were detected using specific anti-SREBP2, anti-LDLR, anti-HMGCAR, and anti- $\beta$ -actin primary antibodies, respectively (A). SREBP2 (B), LDLR (C), and HMGCAR (D) signals were quantified by ImageJ software and normalized with  $\beta$ -actin signals. Bars represent averages of duplicate samples  $\pm$  SEM of three independent experiments. (\*)  $P < 0.05$  and (\*\*)  $P < 0.001$  versus C. P, pepsin peptides; T, trypsin peptides; C, control.



**Figure 5.** LDL uptake after P and T peptide treatments. HepG2 cells ( $3 \times 10^4$ ) were treated with P (0.5, 1.0, and 2.5 mg/mL) and T (0.25, 0.5, and 1.0 mg/mL) peptides for 24 h. LDL-Dylight 549 (10.0  $\mu$ g/mL) was incubated for an additional 2 h. Excess LDL-Dylight 549 was removed, cells were washed two times with PBS, and specific fluorescent LDL uptake was analyzed by Synergy H1 (Biotek). Data points represent averages  $\pm$  SEM of three independent experiments in triplicate. (\*)  $P < 0.05$  and (\*\*)  $P < 0.001$  versus C. P, pepsin peptides; T, trypsin peptides; C, control.

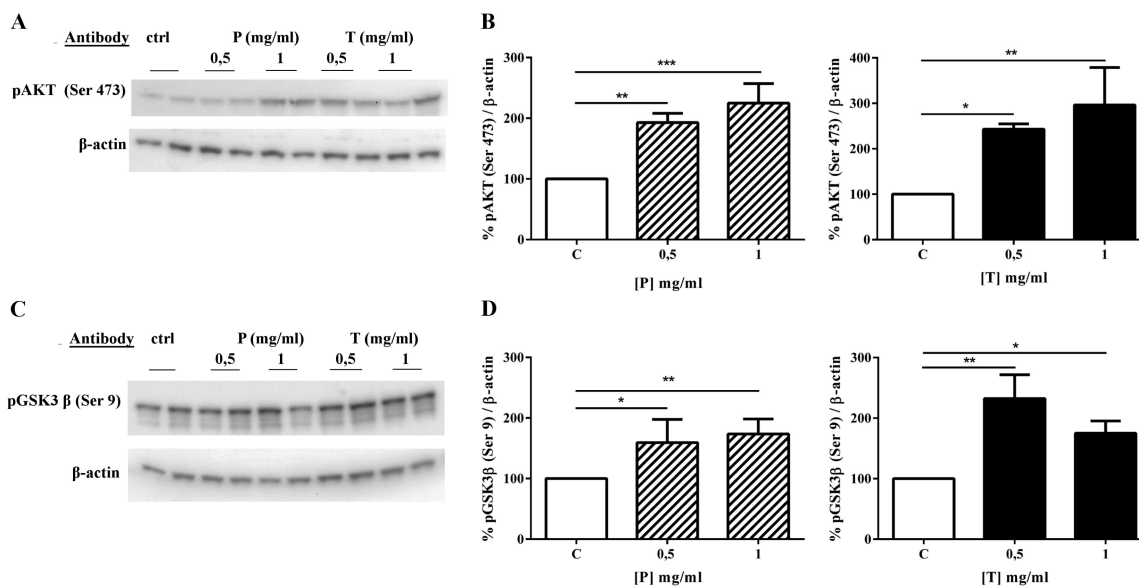
**Lupin Peptides Increase LDL Uptake at the HepG2 Cell Line.** Fluorescent LDL uptake experiments were performed for evaluating the cholesterol-lowering properties of lupin peptides

from a functional point of view. Precisely, the fluorescent LDL uptake was examined in HepG2 cells following a 24 h incubation with P and T peptides. Figure 5 shows that both P and T peptides are able to increase the LDL uptake versus the control in a statistically significant way. In particular, treatment with P peptides at concentrations of 1.0 and 2.5 mg/mL increases the LDL uptake by 42 and 45%, respectively, versus the control, whereas the LDL uptake increase was not statistically significant at 0.5 mg/mL. On the other hand, at concentrations of 0.25, 0.5, and 1.0 mg/mL T peptides significantly raise the LDL uptake by 52, 50, and 70%, respectively, versus the control.

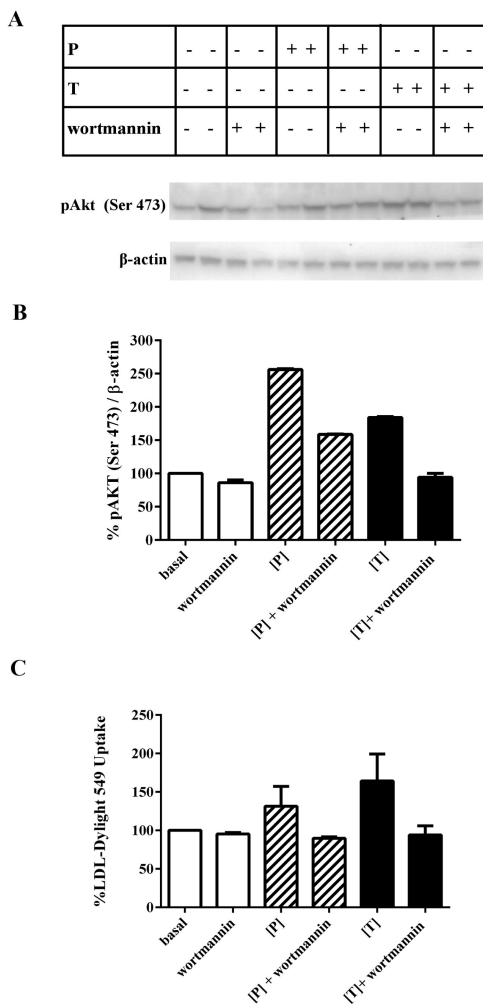
**Lupin Peptides Mediate LDLR-SREBP2 Up-regulation through the Activation of PI3K/Akt/GSK3 $\beta$  Kinases.** To examine the effect of lupin peptides on the activation of Akt and GSK3 $\beta$  (its direct substrate), immunoblot analyses were performed on lysates from treated HepG2 cells using antibodies, which are specific for Akt phosphorylated at serine residue 473 and for GSK3 $\beta$  phosphorylated at serine residue 9. In accordance with the preceding immunoblot experiments, HepG2 cells were treated with P and T peptides at concentrations of 1.0 and 0.5 mg/mL, and the variation of phosphorylated Akt and GSK3 $\beta$  levels was investigated. Figure 6B shows that the treatments with lupin peptides increased the Akt phosphorylation in a dose-dependent manner: by 92 and 125%, respectively, after treatment with P peptides at 0.5 and 1.0 mg/mL and by 144 and 196%, respectively, after treatment with T peptides at 0.5 and 1.0 mg/mL.

Additionally, P peptides mediate a 60% increase of the phosphorylation level of GSK3 $\beta$  at 0.5 mg/mL and a 74% increase at 1.0 mg/mL versus the control. After T peptide treatments, instead, the GSK3 $\beta$  phosphorylation level was increased by 133% at 0.5 mg/mL and by only 75% at 1.0 mg/mL (Figure 6D).

Treatment with 1  $\mu$ M wortmannin, a PI3K inhibitor, inhibited the Akt phosphorylation either in the presence or in the absence of both lupin peptide mixtures. Figure 7A shows a representative immunoblot and the relative intensity of the phospho-Akt bands. The quantification and normalization against actin bands from



**Figure 6.** Effect of P and T peptides on the activation of Akt (Ser473) and GSK3 (Ser9). HepG2 cells ( $1.5 \times 10^5$ ) were treated with 0.5 and 1 mg/mL of P and T peptides for 24 h, respectively. The phosphorylation levels of Akt (Ser473) (A) and GSK3 (Ser9) (C) and  $\beta$ -actin immunoblotting signals were detected using specific anti-phosphoAkt (Ser473), anti-phosphoGSK3 $\beta$  (Ser9), and anti- $\beta$ -actin primary antibodies, respectively. PhosphoAkt signals (B) and phosphoGSK3 $\beta$  (D) were quantified by ImageJ software and normalized with  $\beta$ -actin signals. Bars represent averages of duplicate samples  $\pm$  SEM of three independent experiments. (\*)  $P < 0.05$ , (\*\*)  $P < 0.001$ , and (\*\*\*)  $P < 0.0001$  versus C. P, pepsin peptides; T, trypsin peptides; C, control; pAkt, phosphoAkt; pGSK3 $\beta$ , phosphoGSK3 $\beta$ .



**Figure 7.** Involvement of PI3K/Akt pathway activation in the regulation of the LDLR–SREBP2 pathway through which lupin peptides mediate the cholesterol-lowering effects. HepG2 cells ( $1.5 \times 10^5$ ) were treated with 1.0 and 0.5 mg/mL of P and T peptides in the presence or absence of wortmannin for 24 h, respectively. The phosphorylation level of Akt (Ser473) (A) and  $\beta$ -actin immunoblotting signals were detected using specific anti-phosphoAkt (Ser473) and anti- $\beta$ -actin primary antibodies, respectively. Relative intensity of phosphoAkt signals (B) was quantified by ImageJ software and normalized with  $\beta$ -actin signals. Panel C shows the LDL uptake after treatment with the PI3K inhibitor. HepG2 cells ( $3 \times 10^4$ ) were treated with P (1.0 mg/mL) and T (0.5 mg/mL) peptides in the presence or absence of wortmannin and inhibitor alone for 24 h. LDL-Dylight 549 (10.0  $\mu$ g/mL) was incubated for additional 2 h. Excess LDL-Dylight 549 was removed, and cells were washed two times with PBS. Specific fluorescent LDL uptake was analyzed by Synergy H1 (Biotek). Bars represent averages of duplicate samples  $\pm$  SEM of three independent experiments. pAkt, phosphoAkt; P, pepsin peptides; T, trypsin peptides; C, control.

three different experiments, each performed in duplicate, is shown in Figure 7B. This figure clearly demonstrates that the wortmannin co-incubation with P and T peptides is able to reduce by about 40 and 50% the Akt phosphorylation level, abolishing the Akt phosphorylation induced by lupin peptides.

To confirm the involvement of the PI3K/Akt pathway activation in the regulation of the LDLR–SREBP2 pathway, functional LDL uptake experiments were performed in the presence of wortmannin. As Figure 7C shows, P peptides and T peptides are able to increase the LDL uptake with respect to the basal level, but these uptakes are blocked in the presence of

wortmannin. Precisely, the LDL uptake is blocked at the basal level either in the presence of the inhibitor alone or in the co-incubation with P and T peptides, demonstrating that the LDL uptake induced by lupin peptides is abolished by treatment with this well-known PI3K inhibitor.

## DISCUSSION

As already indicated in the Introduction, some experimental studies<sup>11–14</sup> and a few clinical trials<sup>17–19</sup> gave clear indications that lupin proteins are able to induce hypocholesterolemic effects in vivo. The present investigation at HepG2 cells was aimed at providing some further information on the mechanism through which the lupin peptides may be responsible of the observed activities.

The main findings of this study are the following: (i) T peptides are able to directly interfere with the HMGCoAR activity, whereas P peptides are much less effective. (ii) Both P and T peptides modulate the cholesterol metabolism at HepG2 cell line, through the up-regulation of the pathway involving the LDLR. (iii) Both P and T peptide treatments increase the LDL-uptake. (iv) Activation of the Akt/GSK3 $\beta$  pathway is involved in the up-regulation of the LDLR–SREBP2 pathway.

HMGCoAR is the rate-controlling enzyme of cellular cholesterol biosynthesis pathway, and therefore it constitutes the target of numerous investigations aimed at lowering the rate of cholesterol biosynthesis.<sup>49</sup> Therefore, initially in vitro experiments were performed using the purified catalytic domain of the enzyme with the objective of clarifying whether lupin peptides were able to directly inhibit the activity of HMGCoAR. As shown in Figure 3, T peptides but not P peptides act as enzyme inhibitors.

The LDLR expression and the receptor protein localization at cellular membranes are strictly correlated to the intracellular cholesterol biosynthesis pathway. In fact, the transcription of the LDLR and the genes required for cholesterol and fatty acid synthesis are controlled by membrane-bound transcription factors called SREBPs,<sup>50</sup> and the intracellular cholesterol acts with a negative feedback inhibition mechanism.<sup>51</sup> The SREBP2 isoform is responsible for the LDLR and HMGCoAR transcription, and the SREBP2 maturation is regulated by the intracellular cholesterol homeostasis. Thus, the up-regulation of LDLR represents a useful strategy to control plasma LDL cholesterol levels. Our findings demonstrate that both lupin peptide mixtures are able to up-regulate the LDLR protein levels through an increase of SREBP2 protein. However, a detailed investigation of the LDLR pathway revealed that lupin peptides produce different effects on the HMGCoAR level. In particular, whereas P peptides are able to increase the enzyme protein level in a dose-dependent manner, T peptides up-regulate the enzyme protein levels in a significant way versus the control at 0.5 mg/mL; the increase remains statistically significant, but it is smaller at 1.0 mg/mL, and it is finally completely abolished at 2.5 mg/mL (Figure 4D).

These pieces of evidence suggest that both peptide mixtures modulate the cholesterol metabolism pathway through the induction of LDLR protein levels due to an increase of SREBP2 protein, although their potencies of induction are different.

In agreement with immunoblotting results, the increase of LDLR protein levels leads to an increase of LDL uptake (Figure 5). Our findings suggest that both P and T peptides are able to significantly induce the LDL clearance, and this result is strictly correlated to an increase of LDLR protein level.

Recently, studies have indicated that there are links between the Akt and the SREBP pathways: in fact, emerging evidence

shows that Akt is implicated in the regulation of lipid metabolism through the activation of SREBPs.<sup>45,52–55</sup> Luu and co-workers<sup>41</sup> showed that insulin-like growth factor-1, an inducer of Akt signaling, acutely increases SREBP2 activation. This study provided evidence that Akt contributes to the regulation of cholesterol metabolism through activating SREBP2.

Even if the precise target or targets of Akt remain elusive and not clarified, part of our investigation was dedicated to evaluating the effects of lupin peptides on the PI3K/Akt pathway. Our study provides experimental evidence that either P or T peptides from lupin protein are able to induce increases of Akt and GSK3 $\beta$  phosphorylation levels, which are completely abolished by treatment with a well-known PI3K inhibitor, such as wortmannin. A constitutively active form of Akt activated the LDLR.<sup>53</sup> Our findings clearly support this study, because both P and T peptides increase the LDLR protein levels and induce an increased fluorescent LDL uptake at HepG2 cells. Moreover, after treatment with both lupin peptides in the presence of wortmannin, LDL uptake is blocked versus the P and T treatments alone, demonstrating that the inhibition of PI3K/Akt has general effects on cellular lipid homeostasis, although the precise Akt target or targets are not definitely assigned yet.

In conclusion, this is the first study providing evidence that peptide mixtures obtained by the hydrolysis of lupin proteins are able to modulate the LDLR/SREBP2 pathway with the final effect of an increased LDL uptake. Because, as indicated above, both P peptides and T peptides are complex mixtures, it appears very difficult to sort out which may be the peptides responsible for the observed activities. It is, however, possible to affirm that the diversity in the behaviors of the two peptide mixtures derives from their different compositions. Further work will be necessary to investigate these aspects in detail. This is also the first study to investigate in detail the mechanism of the hypocholesterolemic activity of peptides deriving from a plant protein, because, even in the case of soy, only partial information is available.<sup>26–32</sup>

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

Tables 1S and 2S. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Ideation and design of experiments: C.L. Experiments: biotechnology, C.L. and C.Z.; mass spectrometry analysis, A.D.; peptide preparation, G.M.S.; analysis of data, C.L., C.Z., and A.D.; figure preparation, C.Z.; retrieval of grants, A.A.; manuscript writing, C.L. and A.A.

### ■ Funding

Research was funded by the European Union Seventh Framework Programme (FP7/2007–2013), under Grant Agreement 285819. We are indebted to Regione Lombardia and HPF-Nutraceuticals SRL (Milan, Italy) for the partial funding of a postdoctoral fellowship to C.Z. and to the Carlo Sirtori Foundation (Milan, Italy) for having provided most of the equipment used in this experimentation.

### ■ Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS USED

HMGCoAR, 3-hydroxy-3-methylglutaryl CoA reductase; LDL, low-density lipoprotein; LDLR, LDL receptor; *L. albus*, *Lupinus albus*; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; SREBP2, sterol regulatory element binding protein 2; SCAP, SREBP cleavage-activating protein; ER, endoplasmic reticulum; P peptides, pepsin peptides; T peptides, trypsin peptides; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$

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