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Lupin protein exerts cholesterol-lowering effects targeting PCSK9: From clinical evidences to elucidation of the *in vitro* molecular mechanism using HepG2 cells

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ABSTRACT

PCSK9 inhibition is a novel approach for cholesterol reduction because of its crucial pathophysiological role in cholesterol metabolism. This work aimed at evaluating whether lupin protein/peptides may modulate PCSK9 production. Mild hypercholesterolaemic subjects consumed lupin protein or casein (30 g/day) for 4 weeks. The final level of circulating PCSK9, measured by ELISA, was reduced by 8.5% ($p = 0.0454$) versus baseline value, whereas it remained unchanged in the control group (casein). For investigating the mechanism of action, HepG2 cells were treated with peptic and tryptic peptides from lupin protein: reductions of PCSK9 production and secretion were observed as well as a decrease of hepatic nuclear factor 1-alpha (HNF1-alpha). For the first time, this work provides evidences that lupin protein/peptides may modulate the PCSK9 protein level production and secretion, contributing to explain the beneficial effects observed in animal and human studies and opening a completely new area of investigation on plant proteins.

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1. Introduction

Proprotein convertase (PC) subtilisin/kexintype 9 (PCSK9) is primarily synthesised in the liver and small intestine (Benjannet et al., 2004). After intracellular autocatalytic cleavage of its prodomain, mature PCSK9 is secreted from liver cells (McNutt, Lagace, & Horton, 2007). Animal studies have shown that the

PCSK9 binding to the low density lipoprotein (LDL) receptor (LDLR) targets the receptor for lysosomal degradation, thereby providing a possible mechanism through which PCSK9 may affect cholesterol metabolism (Lo Surdo et al., 2011). In the absence of PCSK9, the hepatic LDLR is shuttled back to the plasma membrane after cholesterol delivery to the lysosome for degradation. PCSK9 binding, instead, prevents this LDLR shuttling and targets it for degradation (Lagace et al., 2006).

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Abbreviations: FBS, foetal bovine serum; HMGCoAR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HNF1-alpha, hepatocyte nuclear factor 1 alpha; LDL-C, LDL cholesterol; PCSK9, proprotein convertase subtilisin/kexintype 9; SREBP-2, regulatory element binding proteins 2

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PCSK9 primarily acts on the LDLR as a circulating plasma protein and several small-scale studies have shown a positive relationship between circulating PCSK9 and LDL cholesterol (LDL-C) levels (Lambert, Sjouke, Choque, Kastelein, & Hovingh, 2012).

High levels of circulating LDL-C accelerate the progression of atherosclerosis and LDL-C lowering is currently considered the most effective strategy in preventing coronary artery disease. Several recent studies have correlated the novel target PCSK9 with parameters directly related to atherosclerosis progression (Giunzioni & Tavori, 2015; Gu & Zhang, 2015; Stein & Raal, 2015; Zhang et al., 2015). The role of circulating PCSK9 in promoting hypercholesterolaemia is beyond dispute and strongly supported by preclinical experiments and clinical trials, where antibodies directed against the LDLR binding site in PCSK9 have effectively reduced LDL-C levels (Roth, McKenney, Hanotin, Asset, & Stein, 2012; Stein et al., 2012).

Although specific drugs, such as statin, are the main solution for the treatment of hypercholesterolaemia, a number of new pharmacological approaches based on the inhibition of PCSK9 function are currently under development (Lambert et al., 2012; Verbeek, Stoekenbroek, & Hovingh, 2015). In this scenario, also natural compounds may have an important preventive role. An example is berberine (Kong et al., 2004), an isoquinoline alkaloid found in the leaves of some species of the genus *Berberis*, which inhibits the progression of hypercholesterolaemia targeting PCSK9 (Cameron, Ranheim, Kulseth, Leren, & Berge, 2008).

Lupin is a grain legume cultivated as a sustainable crop in many countries either for animal or human nutrition. The interest for this seed relies mostly on its high protein content (35–40%). Besides its useful nutritional features, in the last decade numerous investigations have shown that lupin provides interesting health benefits (Arnoldi, Boschin, Zanoni, & Lammi, 2015), particularly in the area of hyperglycaemia control (Bertoglio et al., 2011; Duranti, Consonni, Magni, Sessa, & Scarafoni, 2008; Lovati et al., 2012), hypertension prevention (Boschin et al., 2014; Lee et al., 2009), and cholesterol reduction (Bähr, Fechner, Kiehntopf, & Jahreis, 2015; Marchesi et al., 2008; Sirtori et al., 2004, 2012; Weisse et al., 2010). The protein seems to be relevant in these beneficial effects (Arnoldi et al., 2015).

In the framework of the European project Bioprofibre, some years ago, we have performed a double-blind randomised clinical trial for evaluating the potential hypocholesterolaemic effects of some legume proteins, including lupin protein, versus casein as control protein in mild hypercholesterolaemic patients (Sirtori et al., 2012). This intervention study had a parallel design and was 4 weeks long. The consumption of the dietary bar containing lupin protein (30 g/day) resulted in a significant reduction of total cholesterol (-11.6 mg/dl = -4.2% , $p < 0.05$), whereas no significant cholesterol changes were observed in the subjects consuming the control bars containing casein.

Further studies were dedicated to elucidate the mechanism of action, mainly working on peptides. In particular, we have demonstrated that tryptic and peptic peptides derived from lupin protein are able to interfere with 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) activity, up-regulating the LDLR and sterol regulatory element binding proteins 2 (SREBP-2), and increasing the LDL-uptake in HepG2 cells (Lammi, Zanoni, Scigliuolo, D'Amato, & Arnoldi, 2014).

These results suggested the possibility that lupin protein may also influence the expression of PCSK9. In order to achieve a rapid confirmation of this hypothesis, we decided to measure the level of circulating PCSK9 in the plasma of the subjects of the clinical study described above (Sirtori et al., 2012). The positive results of this preliminary experiment prompted us to undertake an extensive *in vitro* investigation on the mechanism of action through which peptic and tryptic peptides from lupin protein modulate PCSK9 production by targeting its intracellular processing. This mechanistic study was performed using human hepatic HepG2 cells as model system, since hepatocytes are the main source of circulating PCSK9 (Lagace et al., 2006). The same cellular model had been already used by us to characterise the hypocholesterolaemic effects of lupin peptides (Lammi et al., 2014), since it also expresses high levels of LDLR (Tavori et al., 2013).

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, foetal 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, and the antibody against β -actin were bought from Sigma-Aldrich (St. Louis, MO, USA). The antibody against PCSK9 and HNF1-alpha were bought from GeneTex (Irvine, CA, USA). The antibodies against rabbit Ig-HRP, mouse Ig-HRP, phenylmethanesulfonyl fluoride (PMSF), Na-orthovanadate inhibitors were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); the inhibitor cocktail Complete Midi was purchased from Roche (Basel, Swiss). Mini protean TGX pre-cast gel 7.5% and Mini nitrocellulose Transfer Packs were purchased from BioRad (Hercules, CA, USA). The Human Proprotein Convertase 9 Immunoassay (Quantikine ELISA) was bought from R&D System (Minneapolis, MN, USA).

2.2. Brief description of protocol of Bioprofibre clinical study and quantification of PCSK9 by ELISA in the subject plasma

The complete protocol of the clinical study and details of the ethical approval are described in a previous paper (Sirtori et al., 2012). Briefly, participants were recruited at the University Center for Dyslipidaemias of the Niguarda Hospital (Milano, Italy), according to the following criteria: (i) males and postmenopausal females; (ii) participants in primary prevention, non-diabetics; (iii) total cholesterol >220 mg/dL and triglycerides <200 mg/dL. A randomised, double-blind, parallel group design was followed, with total cholesterol as main endpoint. All the candidates underwent a stabilisation period on a hypolipidaemic dietary regimen for 4 weeks. The selected subjects were then randomised into the dietary treatment groups: each group consumed for 4 weeks two bars per day containing 30 g the specific protein source, i.e. casein (control) or a lupin protein isolate prepared by the Fraunhofer Institute IVV (Freising, Germany). At the starting and final visits, blood samples were

collected after an overnight fast. Both serum and ethylenediaminetetraacetic acid (EDTA) plasma were prepared by low-speed centrifugation at 4 °C and stored at –80 °C. Plasma total cholesterol, triacylglycerols, and HDL-cholesterol (HDL-C) were determined with standard enzymatic techniques (Roche Diagnostics) on a Roche Diagnostics Cobas 400 Analyzer. Plasma LDL-C was calculated with the Friedewald's formula. For measuring PCSK9, aliquots of the plasma samples were 20-fold diluted with Calibrator diluent RD5P (provided by the ELISA kit of R&D System, Minneapolis, MN, USA). The experiments were carried out at 37 °C following the manufacturer's instructions. Before starting the assay, human PCSK9 standards (20.0, 10.0, 5.0, 2.5, 1.25, and 0.625 ng/mL) were prepared from the stock solution PCSK9 Standard (40 ng/mL) with serial dilutions (for building the standard curve) and meanwhile 100 µL the Assay Diluent RD1-9 (provided into the kit) were added to each wells. Afterward, standards and samples (50 µL) were pipetted into the wells and the ELISA plate was allowed to incubate for 2 h at RT. Subsequently, wells were washed 4 times with Wash Buffer, and 200 µL of human PCSK9 Conjugate (HRP-labelled anti-PCSK9) was added to each well for a 2 h incubation at RT. Following aspiration, wells were washed 4 times with Wash Buffer provided by the kit. After the last wash, 200 µL of Substrate Solution was added to the wells and allowed to incubate for 30 min at RT. The reaction was stopped with 50 µL of Stop Solution (2 N sulphuric acid) and the absorbance at 450 nm was measured using Synergy H1 (Biotek, Bad Friedrichshall, Germany). The lower limit of detection was 0.096 ng/mL.

2.3. Preparation and analysis of the pepsin and trypsin peptide mixtures

White lupin seeds (*Lupinus albus* cultivar Ares) were provided by Terrena (Matrignè-Ferchaud, France). The total protein extract was obtained and analysed as previously reported (Lammi et al., 2014). In brief, 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. After centrifugation at 6500 g, for 20 min at 4 °C, the supernatant was dialysed against 100 mM Tris-HCl buffer pH 8.2 for 24 h at 4 °C. After assessing the protein concentration by Bradford assay, the total protein extract was dissolved in Tris-HCl buffer 100 mM at pH 8, then the pH was adjusted to the optimal hydrolysis conditions for each enzyme (pH = 2 for pepsin and = 8 for trypsin) by adding 1 M NaOH or 1 M HCl. After 18 h incubation and enzyme inactivation, the mixtures were ultra-filtered through 3000 Da cut-off centrifuge filters (Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 12,000 g for 30 min at 4 °C. The analyses were conducted on a nano-chromatographic system, UltiMate 3000 RSLCnano System (Thermo Scientific, Waltham, MA, USA). The peptide mixtures were loaded on a reversed-phase trap column (Acclaim PepMap100, C18, 100 Å, 100 µm i.d. × 2 cm, Thermo Scientific) for the cleanup and pre-concentration, then separated on fused silica reverse-phase column (picoFrit column, C18, 2.7 µm, New Objective), eluting 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 connected to a 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. The results of the analysis are reported in detail in a previous paper (Lammi et al., 2014).

2.4. Cell line culture

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, 100 µg/mL streptomycin (complete growth medium) and incubated at 37 °C under 5% CO₂ 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.

2.5. Western blot analysis

1.5×10^5 HepG2 cells/well (24-well plate) were treated with 1.0 and 2.5 mg/mL of peptic and 0.5 and 1.0 mg/mL of tryptic peptides for 24 h. After each treatment, the medium of each well was collected in an ice-cold microcentrifuge tube and processed for the PCSK9 immunoassay. Meanwhile the cells were scraped in 40 µL ice-cold lysis buffer (RIPA buffer + inhibitor cocktail + 1:100 PMSF + 1:100 Na-orthovanadate) and transferred in an ice-cold microcentrifuge tube. After centrifugation at 16,060 g for 15 min at 4 °C, the supernatant was recovered and transferred in a new ice-cold tube. Total proteins were quantified by the Bradford method and 50 µg of total proteins loaded on a pre-cast 7.5% sodium dodecyl sulphate–polyacrylamide (SDS-PAGE) gel at 130 V for 45 min. Subsequently, the gel was transferred to a nitrocellulose membrane (Mini Nitrocellulose Transfer Packs), using a Trans-blot Turbo at 1.3 A, 25 V for 7 min. Target proteins, on milk blocked membrane, were detected by primary antibodies as follows: anti-PCSK9, anti-HNF1-alpha, and anti-β-actin. Secondary antibodies conjugated with HRP and a chemiluminescent reagent were used to visualise target proteins and their signal was quantified using the Image Lab Software (Bio-Rad). The internal control β-actin was used to normalise loading variations.

2.6. Quantification of excreted PCSK9 in cell culture experiments by ELISA

The supernatants collected from HepG2 cells were centrifuged at 600 g for 10 min at 4 °C. They were recovered and diluted with the ratio 1:10 with DMEM in a new ice-cold tube. PCSK9 was quantified by ELISA (R&D System, Minneapolis, MN, USA) using the same kit and methodology described above for the quantification in the plasma of the subjects of the clinical study (Section 2.2).

2.7. Statistically analysis

Statistical analyses were carried out by one-way ANOVA using the software Prism 6 (GraphPad, La Jolla, CA, USA) followed by Dunnett's test. Values were expressed as means ± SEM; p-values < 0.05 were considered to be significant.

3. Results

3.1. Lupin protein reduce the plasma levels of PCSK9 in mild hypercholesterolaemic subjects

The control group (casein bar) and treatment group (lupin protein bar) of the clinical trial (Sirtori et al., 2012) were composed by 19 and 20 individuals, respectively. For 4 weeks, they consumed two dietary bars corresponding to a total amount of 30 g protein per day. The average baseline and final values of the lipids and PCSK9 plasma levels are reported in Table 1, whereas HDL-cholesterol and total triglyceride levels remained essentially unchanged in both groups, a significant total cholesterol reduction (-11.6 mg/dL = -4.2% , $p < 0.05$) and a small non-significant LDL-cholesterol one were observed in the lupin group, but not in the control group. Additionally, a significant reduction of PCSK9 plasma level was detected in the treatment group, whereas only a very small non-statistically significant change was observed in the control group. In particular, when the data were compared by ANOVA with the Dunnett's test, followed by adjustment for baseline values, the reduction of the plasma PCSK9 levels of the lupin group was equal to 8.5% ($p = 0.0454$) versus the control group.

3.2. Preparation and analysis of the peptide mixtures

A total protein extract from lupin seed was hydrolysed separately with pepsin and trypsin to produce peptic peptides and tryptic peptides. Analysis by nano LC-MS/MS permitted to identify more than 2000 peptides in the pepsin digested mixture and about 3000 in the trypsin digested one (Lammi et al., 2014). By consulting the Uniprot_viridiplantae database using the Mascot software, it was possible to assign only a small number of peptides to known lupin proteins, probably due to the very incomplete sequencing of lupin proteins. Most peptides belong to the main lupin storage proteins, by far the most abundant in the total protein extract as shown by SDS-PAGE.

In the tryptic sample (Table 2a), 12 peptides were assigned to *L. albus* vicilin-like protein (Q53HY0), 10 peptides to *L. albus*

beta-conglutin precursor (Q6EBC1), 4 peptides to *Lupinus angustifolius* conglutin beta (B0YJF8), 4 peptides to *L. angustifolius* conglutin-beta (Q53I55), and 2 to *L. albus* conglutin-delta seed storage protein precursor (Q99235). Moreover, two peptides were assigned to another plant protein, i.e. *Zea mays* actin partial (ADF3). In the peptic hydrolysate (Table 2b), 21 peptides were assigned to *L. albus* vicilin-like protein (Q53HY0), 18 peptides to *L. albus* beta-conglutin (Q6EBC1), 7 peptides to *L. angustifolius* conglutin-alpha 3 (F5B8V8), and 8 peptides to *L. albus* conglutin-gamma (Q9FSH9).

3.3. Lupin peptides reduce the protein levels of PCSK9 and hepatocyte nuclear factor 1 alpha (HNF1-alpha) in HepG2 cells

HepG2 cells were treated for 24 h with peptic and tryptic peptides at the concentrations of 1.0–2.5 mg/mL and 0.5–1.0 mg/mL, respectively. Using immunoblotting technique, two bands were detected: the former corresponded to the precursor ~ 75 kDa PCSK9 (PCSK9-P), the latter to the mature ~ 62 kDa PCSK9 (PCSK9-M). Immunoblotting experiments showed that the treatments with both lupin peptide mixtures reduced the PCSK9 protein levels (Fig. 1). In particular, lupin peptic peptides (1.0 and 2.5 mg/mL) reduce the precursor PCSK9 protein level by 66% and 58% versus the untreated sample, while tryptic lupin peptides (0.5 and 1.0 mg/mL) reduce the precursor PCSK9 protein levels by 61% and 53% versus the control, respectively, at each treatment concentration (Fig. 1B).

In the same experiments, also the protein levels of mature PCSK9-M were measured by immunoblotting. Fig. 1C shows that both peptic and tryptic peptides are able to decrease the protein level of PCSK9-M versus the untreated sample. In particular, at 1.0 and 2.5 mg/mL, respectively, peptic peptides mediate 52% and 58% reductions of the PCSK9-M protein, whereas, at 0.5 and 1.0 mg/mL, tryptic peptides mediate 61% and 44% decreases versus the untreated sample (Fig. 1C).

Both peptic and tryptic peptides affected also the protein levels of HNF-1 alpha (Fig. 2): peptic peptides decreased it by 28% at 1 mg/mL and 25% at 2.5 mg/mL versus the untreated sample, whereas the treatment with tryptic peptides led to a significant 30% reduction of the HNF1-alpha protein production at 1.0 mg/mL, but not at 0.5 mg/mL.

3.4. Lupin peptides reduce the secretion of mature PCSK9 by human hepatic HepG2 cells

The same cells were treated with peptic peptides (1.0 and 2.5 mg/mL) and tryptic peptides (0.5 and 1.0 mg/mL) for 24 h. On the second day, the cell culture medium was collected and the effects of lupin peptide treatments on the capacity of HepG2 cells to secrete PCSK9-M were assessed using the ELISA kit. Fig. 3 clearly indicates that both peptic and tryptic lupin peptides significantly reduced the secretion of PCSK9-M by about one third vs. the untreated samples. More in detail, untreated HepG2 cells secreted 141 ng/mL of PCSK9-M, whereas after treatment with peptic lupin peptides (1.0 and 2.5 mg/mL), they secreted 94.3 and 97.3 ng/mL of PCSK9 and, after treatment with tryptic lupin peptides (1.0 and 0.5 mg/mL), they secreted 94.3 and 93.6 ng/mL PCSK9-M, respectively.

Table 1 – Effect of lupin protein consumption on lipid parameters and PCSK9 plasma levels in mild hypercholesterolaemic patients: data are expressed as mean \pm SD.

Parameter		Casein	Lupin protein
Total cholesterol (mg/dL) ^a	Baseline	272.4 \pm 34.4	274.0 \pm 40.0
	4 weeks	275.2 \pm 26.9	262.4 \pm 40.8*
LDL-cholesterol (mg/dL) ^a	Baseline	188.8 \pm 36.5	188.2 \pm 35.4
	4 weeks	193.7 \pm 26.7	182.6 \pm 40.6
HDL-cholesterol (mg/dL) ^a	Baseline	57.0 \pm 12.4	56.0 \pm 13.9
	4 weeks	57.6 \pm 12.8	54.7 \pm 15.7
Triglycerides (mg/dL) ^a	Baseline	126.1 \pm 45.4	145.6 \pm 70.9
	4 weeks	115.2 \pm 34.6	126.5 \pm 59.6
PCSK9 (ng/mL)	Baseline	78.8 \pm 17.3	82.9 \pm 22.2
	4 weeks	75.3 \pm 14.0	75.9 \pm 22.4*

* $p < 0.05$ vs. baseline.

^a Selected data from a previous paper (Sirtori et al., 2012).

Table 2 – (a) Lupin peptides identified by mass spectrometry in the tryptic hydrolysate. (b) Lupin peptides identified by mass spectrometry in the peptic hydrolysate.

Accession number	Protein name	Mascot protein score	Protein mass	N. peptides	m/z	z	Mr	Peptide delta	Ion score	Peptide sequence
(a)	Q53HY0 Vicilin-like protein [<i>Lupinus albus</i>]	969	61994	12	419.35	2	836.40	0.27	40	YEEIQR
					1051.58	1	1050.50	0.07	32	SNEPIYSNK
					526.62	2	1050.50	0.73	27	SNEPIYSNK
					688.15	2	1373.63	0.66	47	YGNFYEITPDR
					1462.62	1	1462.69	−0.08	47	ELTFPGSAEDIER
					732.62	2	1462.69	0.54	63	ELTFPGSAEDIER
					762.62	2	1522.66	0.56	76	EQEQQQGSPSYSR
					1557.63	1	1556.79	−0.17	102	HSDADYVLVVLNGR
					779.47	2	1556.79	0.12	63	HSDADYVLVVLNGR
					791.66	2	1580.75	0.56	61	GQEQSHQDEGVIVR
					823.03	2	1643.69	0.35	76	QQDEQEVEEVEVR
					863.99	2	1726.00	−0.04	28	IVEFQSKPNTLILPK
					576.40	3	1726.00	0.18	48	IVEFQSKPNTLILPK
					932.71	2	1863.85	−0.45	94	ILGNEDQEYEEQR
	840.60	3	2518.31	0.46	33	LSEGDIFVIPAGYPISVNASSNLR				
	1090.84	3	3268.66	0.83	71	NPQVQDLDISLTFTEINEGALLPHYNSK				
	Q6EBC1 Beta-conglutin precursor [<i>Lupinus albus</i>]	917	62092	10	419.35	2	836.40	0.27	40	YEEIQR
					1051.58	1	1050.50	0.07	32	SNEPIYSNK
					526.62	2	1050.50	0.73	27	SNEPIYSNK
					688.15	2	1373.63	0.66	47	YGNFYEITPDR
					762.62	2	1522.66	0.56	76	EQEQQQGSPSYSR
1557.63					1	1556.79	−0.17	102	HSDADYVLVVLNGR	
779.47					2	1556.79	0.12	63	HSDADYVLVVLNGR	
1568.69					1	1567.84	−0.16	64	INEGALLPHYNSK	
784.95					2	1567.84	0.05	59	INEGALLPHYNSK	
863.99					2	1726.00	−0.04	28	IVEFQSKPNTLILPK	
576.40					3	1726.00	0.18	48	IVEFQSKPNTLILPK	
874.70					2	1746.84	0.55	65	AVNELTFPGSAEDIER	
932.71					2	1863.85	−0.45	94	IILGNEDQEYEEQR	
1034.78					3	3100.55	0.77	72	YGNFYEITPDRNPQVQDLNISLTYIK	
Q99235 Conglutin-delta seed storage protein precursor [<i>Lupinus albus</i>]	573	17641	2	687.93	2	1373.61	0.24	60	IQQQEEEEEGR	
				934.22	2	1866.93	−0.51	56	QEEQLLEQELNLR	
Q53155 Legumin-like protein [<i>Lupinus albus</i>]	399	58830	4	638.61	3	1912.87	−0.06	49	HNIGESTSPDAYNPQAGR	
				957.68	2	1912.87	0.47	78	HNIGESTSPDAYNPQAGR	
				966.12	2	1929.75	0.48	102	HGREEEEEEEEDER	
				1264.26	2	2526.22	0.29	66	LNALPDNTVQSEAGTIETWNP	
				843.21	3	2526.22	0.37	21	LNALPDNTVQSEAGTIETWNP	
BOYJF8 Conglutin-beta [<i>Lupinus angustifolius</i>]	115	61490	4	1591.41	2	3180.45	0.35	100	EGGQQQQQEGGNVLSGFDDEFLEEALSVNK	
				1061.44	3	3180.45	0.84	27	EGGQQQQQEGGNVLSGFDDEFLEEALSVNK	
				419.35	2	836.40	0.27	40	YEEIQR	
				611.40	3	1831.78	−0.61	21	QQDEQEVEEVEVRR	
				840.60	3	2518.31	0.46	33	LSEGDIFVIPAGYPISVNASSNLR	
ADF3 Actin, partial [<i>Zea mays</i>]	90	37282	2	1090.84	3	3268.66	0.83	71	NPQVQDLDISLTFTEINEGALLPHYNSK	
				874.37	2	1746.88	−0.16	90	SYELPDGQVITIGAER	
				652.78	3	1955.04	0.28	27	VAPEEHPTLLTEAPLNPK	
(b)	Q53HY0 Vicilin-like protein [<i>Lupinus albus</i>]	434	61994	21	667.23	1	666.29	−0.07	39	SEGDIF
					695.29	1	694.35	−0.07	32	DISLTF
					415.56	2	828.38	0.73	44	SNKYGNF
					958.32	1	957.49	−0.18	21	AIPINNPY
					505.05	2	1007.50	0.59	32	IKNQQQSY
					1105.36	1	1104.56	−0.21	31	AIPINNPYF
					553.34	2	1104.56	0.11	49	AIPINNPYF
					578.54	2	1154.57	0.50	32	IKNQQQSYF
					1169.52	1	1168.61	−0.10	36	ILNPDDNQKL
					595.27	2	1188.63	−0.10	25	LPHYNSKAIF
					1189.58	1	1188.63	−0.06	38	LPHYNSKAIF
					651.77	2	1301.71	−0.19	27	LLPHYNSKAIF
					652.33	2	1302.57	0.08	62	YPSSTKDQQSY
					454.46	3	1359.69	0.66	35	SRRQRNPYHF

Table 2 – (continued)

Accession number	Protein name	Mascot protein score	Protein mass	N. peptides	m/z	z	Mr	Peptide delta	Ion score	Peptide sequence
Q6EBC1	Beta-conglutin [Lupinus albus]	141	62092	18	681.19	2	1359.69	0.67	32	SRRQRNPYHF
					688.58	2	1375.73	-0.60	56	IVVVGEGNGKYEL
					725.88	2	1449.64	0.10	44	YPSSTKDQQSYF
					763.10	2	1523.76	0.44	43	EITPDRNPQVQDL
					518.59	3	1552.91	-0.17	23	RVVKLAIPINPGY
					844.31	2	1686.82	-0.21	32	YEITPDRNPQVQDL
					844.72	2	1687.80	-0.38	24	RSNEPIYSNKYGNF
					851.24	2	1699.98	0.49	61	RVVKLAIPINPGYF
					865.24	2	1727.73	0.74	47	YDFYPSSTKDQQSY
					1114.25	2	2226.09	0.39	120	TKYAQSSSGDKKPSQSGPFNL
					667.23	1	666.29	-0.07	39	SEGDFI
					415.56	2	828.38	0.73	44	SNKYGNF
					429.48	2	856.50	0.44	54	IKINEGAL
					958.32	1	957.49	-0.18	21	AIPINPGY
					505.05	2	1007.50	0.59	32	IKNQQSY
					1105.36	1	1104.56	-0.21	31	AIPINPGYF
					578.54	2	1154.57	0.50	32	IKNQQSYF
					1169.52	1	1168.61	-0.10	36	ILNPDDNQKL
					603.69	2	1204.62	0.73	22	LPHYNSKAIY
					652.33	2	1302.57	0.08	62	YPSSTKDQQSY
454.46	3	1359.69	0.66	35	SRRQRNPYHF					
725.88	2	1449.64	0.10	44	YPSSTKDQQSYF					
763.10	2	1523.76	0.44	43	EITPDRNPQVQDL					
518.59	3	1552.91	-0.17	23	RVVKLAIPINPGY					
844.31	2	1686.82	-0.21	32	YEITPDRNPQVQDL					
844.72	2	1687.80	-0.38	24	RSNEPIYSNKYGNF					
851.24	2	1699.98	0.49	61	RVVKLAIPINPGYF					
865.24	2	1727.73	0.74	47	YDFYPSSTKDQQSY					
F5B8V8	Conglutin-alpha [Lupinus angustifolius]	73	67683	7	703.36	1	702.37	-0.02	28	VVPQNF
					797.22	1	796.34	-0.13	33	SGFDPQF
					816.38	1	815.45	-0.09	22	LVVPQNF
					854.49	1	853.50	-0.02	21	RGIPAEVL
					1029.57	1	1028.53	0.03	23	VIPPGTPYW
					639.13	2	1275.61	0.64	24	YRNGIYAPHW
					647.15	2	1292.64	-0.36	62	VIPPGTPYWTY
					354.39	2	706.34	0.42	36	FSHFGL
Q9FSH9	Conglutin-gamma [Lupinus albus]	50	49872	8	719.40	1	718.33	0.07	28	FDLNNP
					422.88	2	843.52	0.21	44	VKIPQFL
					619.18	2	1235.64	0.70	31	TPLTISKQGEY
					633.73	2	1264.69	0.76	35	VDGGVHTRAGIAL
					708.72	2	1414.77	0.66	50	DTKKISGGVPSVDL
					726.97	2	1451.81	0.11	46	LTQKGLPNNVQ GAL
					678.14	3	2032.11	-0.71	24	VLPQQDASTKHLHWGNIL

4. Discussion

In recent years, many studies have extensively improved our understanding of the (patho)physiological role of PCSK9 in human biology (Giunzioni & Tavori, 2015; Gu & Zhang, 2015; Lambert et al., 2012). PCSK9 plays a pivotal role in the regulation of the LDLR activity on the hepatic cell surface, because it promotes its degradation and prevents its recycling to the cell membrane. Consequently, it has become a novel target for lipid-lowering therapy and numerous pharmacological approaches to inhibit PCSK9 function are currently under investigation. One possibility is the development of agents that interfere with LDLR binding by targeting PCSK9 in the circulation, as shown with several monoclonal antibodies (Koren et al., 2012), small peptides (Shan et al., 2008; Zhang et al., 2014), and adnectins. Very recently, the American Food and Drug Ad-

ministration has approved the monoclonal antibody evolocumab (Shantha & Robinson, 2015), for the treatment of patients who are unable to keep their LDL-C under control with current treatment options. A second approach is to reduce hepatic PCSK9 synthesis through gene silencing with small interfering RNA (siRNA) or antisense oligonucleotides (Fitzgerald et al., 2014; Lindholm et al., 2012). The third approach, which has not reached clinical development yet, involves inhibition of PCSK9 production by targeting its intracellular processing (Lambert et al., 2012).

Berberine is a naturally occurring alkaloid with cholesterol-lowering properties, present in the formulation of Armolipid and Armolipid Plus (Ruscica et al., 2014), two commercial dietary supplements. Experimental evidences indicate that berberine decreases the PCSK9 mRNA expression and increases LDLR *in vitro* and in animal studies (Cameron et al., 2008; Kong et al., 2004), through the down-regulation of the HNF1-alpha protein

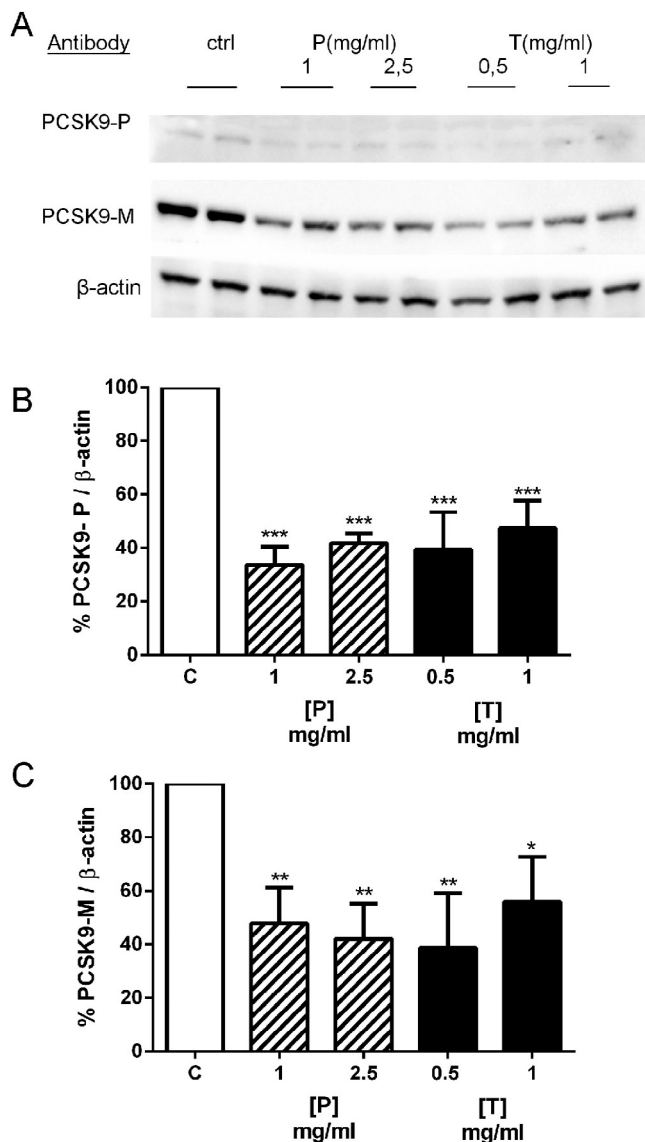


Fig. 1 – Effects of peptic and tryptic lupin peptides on PCSK9 protein levels. HepG2 cells (1.5×10^5) were treated with 1.0 and 2.5 mg/mL of peptic peptides (P) and 0.5 and 1.0 mg/mL of tryptic peptides (T) for 24 h. PCSK9 and β -actin immunoblotting signals were detected using specific anti-PCSK9 and anti- β -actin primary antibodies, respectively. PCSK9-P and PCSK9-M represent the proprotein and the cleaved mature form of PCSK9, respectively (A). PCSK9-P (B) and PCSK9-M (C) signals were quantified by ImageLab software (Bio-Rad) and normalised with β -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 untreated sample (C).

expression (Dong, Li, Singh, Cao, & Liu, 2015). In a similar way, curcumin suppression of PCSK9 expression is associated with increases in cell-surface LDLR expression and activity in HepG2 cells as well as a reduction of nuclear abundance of HNF1-alpha, with a distinct molecular mechanism compared to statins (Tai et al., 2014).

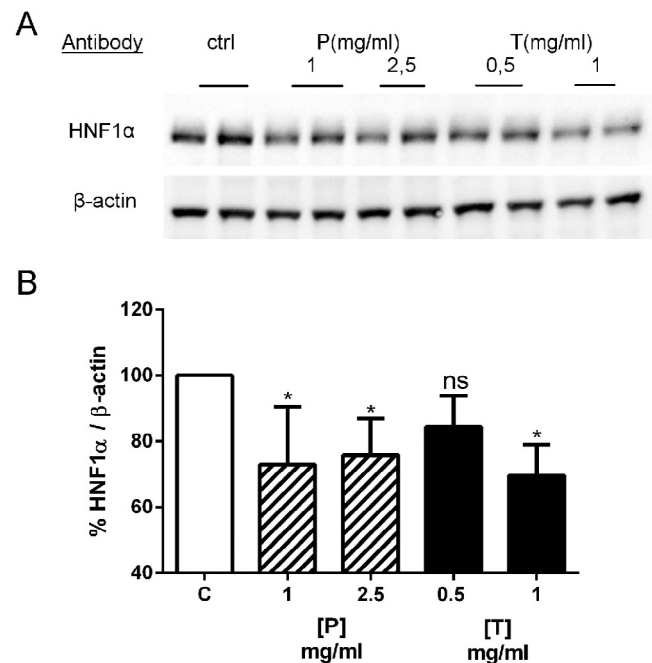


Fig. 2 – Effects of peptic (P) and tryptic (T) lupin peptides on HNF1-alpha protein levels. HepG2 cells (1.5×10^5) were treated with 1.0 and 2.5 mg/mL of peptic peptides (P) and 0.5 and 1 mg/mL of tryptic peptides (T) for 24 h. HNF1-alpha and β -actin immunoblotting signals were detected using specific anti-HNF1-alpha and anti- β -actin primary antibodies, respectively. HNF1-alpha signals (A) were quantified by ImageLab software (Bio-Rad) and normalised with β -actin signals. Bars represent averages of duplicate samples \pm SEM of three independent experiments (B). * $p < 0.05$ versus untreated sample (C).

Up to now, the most common pharmacological strategy for the treatment of hypercholesterolaemia is based on statins, which function by inhibiting HMGCoAR, the rate-limiting enzyme in cholesterol synthesis, thereby elevating the LDLR expression to increase the LDL particle uptake from the circulation (Goldstein & Brown, 2009). However, some patients are insensitive to statin treatment or experience serious adverse effects (Pirillo & Catapano, 2015). Furthermore, statins increase the expression of PCSK9 (Awan et al., 2012), thereby counteracting their beneficial effects.

Using HepG2 cells, we have recently demonstrated that the hypocholesterolaemic effects of peptides deriving from the hydrolysis of lupin protein are based on the inhibition of HMGCoAR activity (Lammi et al., 2014). Similar to statins, the consequent reduction of intracellular cholesterol levels leads to SREBP-2 activation, which in turn increases the expression of LDLR. The consequent improved LDLR activity leads to an enhanced ability of HepG2 cells to uptake extracellular LDL with a final hypocholesterolaemic effect. In this context, this study represents a major innovation, since it provides either clinical indications that a lupin diet decreases the plasma levels of PCSK9 or *in vitro* evidences that lupin peptides may positively influence intracellular PCSK9 processing in hepatocytes.

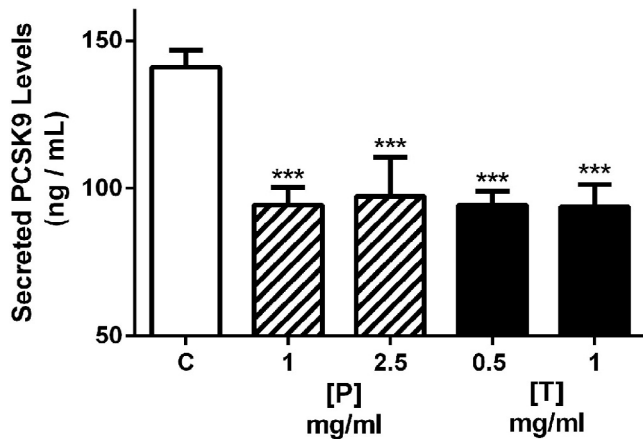


Fig. 3 – Analysis of secreted PCSK9 levels by HepG2 cells after peptic and tryptic lupin peptide treatments. HepG2 cells (1.5×10^5) were treated with 1.0 and 2.5 mg/mL of peptic peptides (P) and 0.5 and 1.0 mg/mL of tryptic peptides (T) for 24 h. After each treatment, the medium was collected and the secreted PCSK9 levels were measured by ELISA. This assay employs the quantitative sandwich enzyme immunoassay techniques. A calibration curve was built using a recombinant human PCSK9. The absorbance of each reaction was measured at 450 nm using the Synergy H1 fluorescent plate reader from BioTek. Data points represent averages \pm SEM of three independent experiments in duplicate. *** $p < 0.0001$ versus untreated sample (C).

To the best of our knowledge, this is the first study showing that the consumption of lupin protein (30 g/day) decreases the plasma PCSK9 levels in moderately hypercholesterolaemic individuals. This means that the potential health benefits of consuming lupin protein may derive not only from the reduction of total cholesterol and LDL-cholesterol (Bähr et al., 2015; Sirtori et al., 2012), but also from the decrease of the PCSK9 plasma levels. This fact is very relevant, since PCSK9 plasma levels correlates with the incidence of cardiovascular disease (CVD) events in humans (Shantha & Robinson, 2015) and are predictive of recurrent clinical events in patients with stable CVD treated with low-dose atorvastatin (Ridker, Rifai, Bradwin, & Rose, 2016).

Furthermore, this work has elucidated some relevant differences between the mechanism of action of lupin and statins. In fact, although lupin peptides apparently inhibit the activity of HMGCoAR as statins (Lammi et al., 2014), surprisingly they do not increase the plasma level of PCSK9 (Table 1), a main drawback of statins.

An important aspect of the PCSK9-LDLR pathway in mediating LDL clearance is that their transcription is coordinately regulated by sterols through a common SRE motif embedded in their gene promoters and is co-induced by current cholesterol lowering drugs, such as statins, through activation of SREBPs (Dubuc et al., 2004; Horton et al., 2003; Jeong et al., 2008). Statin treatment increases the transcription of both LDLR and PCSK9 (Dubuc et al., 2004). This undesirable

inducing effect of statins on PCSK9 transcription is increasingly recognised as a major limitation to their therapeutic efficacy in further lowering plasma LDL-C. While in a preceding paper (Lammi et al., 2014) we have demonstrated that either peptic or tryptic peptides from lupin protein increase the LDLR protein levels and activity through the up-regulation of SREBP-2, here we show that the same peptides also reduce the 692-amino acid precursor (~75 kDa) PCSK9 protein levels with the consequence of a reduction of the mature enzyme (~62 kDa) (Fig. 1).

A general picture of the molecular mechanism through which lupin peptides may induce the cholesterol-lowering effect in hepatocytes is schematically shown in Fig. 4. Pro-PCSK9 undergoes autocatalytic intramolecular processing between the Q152 and S153 residues in the endoplasmic reticulum to form the mature enzyme (Benjannet et al., 2004). The cleavage of the prodomain is required for PCSK9 maturation and secretion (Li et al., 2007; McNutt et al., 2007). In agreement with this consideration and with our clinical results, our findings suggest that the lupin peptide treatment affects the secretion of mature PCSK9 in the culture medium of HepG2 cells (Fig. 3). In order to elucidate the mechanism of action through which lupin peptides positively affect the LDLR pathway reducing the PCSK9 protein levels and secretion, a crucial aspect is the regulation of PCSK9 transcription. In particular, several transcription factors, such as SREBPs and HNF-1, have been identified as transcriptional activators of PCSK9 gene expression (Dong et al., 2010; Horton et al., 2003; Li et al., 2009). PCSK9 and LDLR both contain functional sterol regulatory elements (SREs) in their promoters that respond to change in intracellular cholesterol levels through the activation of the SREBP pathway (Dubuc et al., 2004; Maxwell, Soccio, Duncan, Sehayek, & Breslow, 2003). However, since the HNF1 binding site is unique to the PCSK9 promoter and is not present in the LDLR promoter, modulations of PCSK9 transcription through HNF1 sequence will not affect LDLR gene expression. Thus, the HNF1 binding site represents a divergent point to disconnect the co-regulation of PCSK9 with LDLR and other SREBP target genes (Dong et al., 2015). Indeed, in this study, we have shown that cholesterol-lowering lupin peptides decrease hepatic PCSK9 and secreted PCSK9 concentrations without affecting LDLR protein levels and other SREBP-2 target genes (such as HMGCoAR) and also the down-regulation of HNF1- α protein content in HepG2 cells has been observed (Fig. 2).

For the first time, this investigation provides evidence that the inhibition of PCSK9 production and secretion is a key effect of lupin protein, which contributes to its hypocholesterolaemic property. Furthermore, in the framework of a research aimed at a deep comprehension of the hypocholesterolaemic mechanism of action of lupin protein, these findings help in explaining the beneficial effects observed in the human study, since also a significant reduction of plasma PCSK9 levels has been observed (Sirtori et al., 2012). Our results provide new scientific evidences supporting the use of lupin protein as an ingredient for developing innovative functional foods and open a new area of research on plant proteins in general (Braithwaite et al., 2014; Girgih et al., 2014; Patten, Abeywardena, Head, & Bennett, 2012).

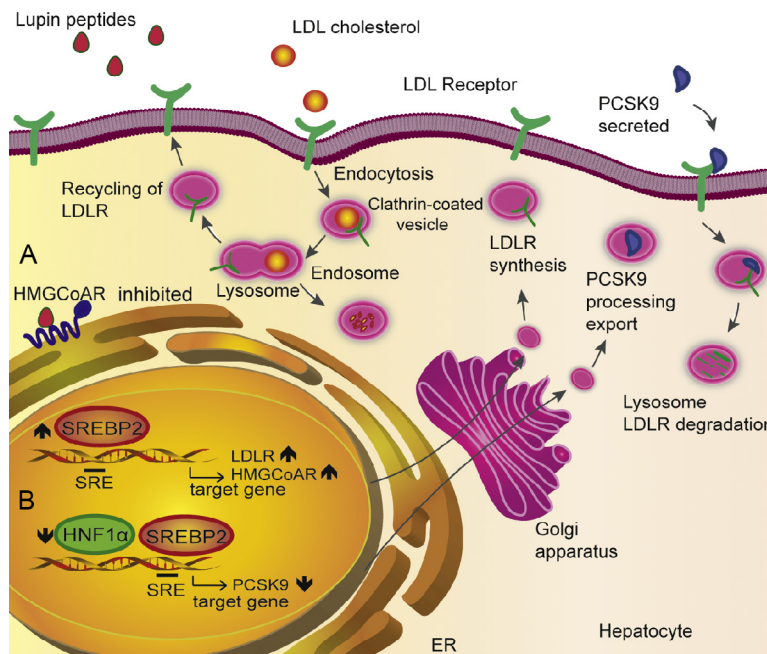


Fig. 4 – Hypocholesterolaemic mechanism of action mediated by lupin peptides in human hepatic HepG2 cells. Upon cell penetration, peptic and tryptic lupin peptides act as competitive inhibitors of HMGCoAR, leading to a reduction of intracellular cholesterol synthesis. When intracellular cholesterol level decreases, the transcription factor SREBP2 is activated and LDLR and HMGCoAR genes are transcribed with subsequent increase of LDLR and HMGCoAR protein levels and localization of LDLR in plasma membrane (A). In parallel, in a synergic way, lupin peptides reduce the PCSK9 protein level production and secretion. In particular, through the reduction of HNF1- α protein level, they lead to a decrease of intracellular precursor and mature PCSK9 protein levels. In agreement, the PCSK9 down-regulation is translated in a reduction of HepG2 cell ability to secrete mature PCSK9 in the extracellular medium, with the consequent stabilisation of active LDLR on hepatic cellular membrane (B). For this reason, the distinct modulation of the two pathways leads to hypocholesterolaemic effects through an improved and synergic activity of LDLR, which can bind and carry the extracellular LDL in HepG2 cells.

Author contribution

CL: ideation, experiment design, and manuscript writing; CZ: experimentation and figure preparation; LC: supervision of the clinical study; AA: manuscript writing and grant retrieval.

Conflict of interest

The authors declare no conflict of interest.

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