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Cholesterol-lowering effect of dietary *Lupinus angustifolius* proteins in adult rats through regulation of genes involved in cholesterol homeostasis

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ABSTRACT

In the absence of a clear indication from previous studies, a rat study was designed to evaluate a possible hypolipidaemic effect of *Lupinus angustifolius* (blue lupin) proteins. Rats were fed for 28 days Nath's hypercholesterolaemic diets containing 20% casein or blue lupin proteins. After 14 and 28 days of dietary treatment, blue-lupin-fed rats had markedly lower plasma total cholesterol levels than rats fed casein (−53.0% and −55.3%, respectively, $p < 0.0005$). No significant differences were instead observed for triglyceride and HDL-cholesterol levels between the two groups. Lupin-protein-fed rats displayed higher hepatic mRNA levels of SREBP-2, a major transcriptional regulator of intracellular cholesterol levels, and CYP7A1, the rate-limiting enzyme in bile acid biosynthesis ($p < 0.05$). In conclusion, the present study demonstrates a marked cholesterol-lowering activity of proteins from *L. angustifolius* in rats. Moreover, blue lupin proteins appear to affect cellular lipid homeostasis by up-regulating SREBP-2 and CYP7A1 genes.

1. Introduction

Interest in vegetable, particular leguminous, proteins has grown considerably in the past years. Lupin proteins provide a potentially important dietary protein source addressed to cardiovascular benefit, since the amino acid content of lupin seeds is very similar to that of the more extensively studied soy, with the advantage of a much lower concentration of isoflavones (Katagiri, Ibrahim, & Tahara, 2000).

Four Mediterranean lupin species (*Lupinus albus*, *Lupinus angustifolius*, *Lupinus luteus*, and *Lupinus mutabilis*) are cultivated for nutrition and they are referred to as sweet lupin, since they all contain small amounts of toxic alkaloids vs. the bitter variety.

Except for one study in rats fed low protein amounts of the legume (Bettzieche, Brandsch, Weisse et al., 2008), previous investigations have generally shown that protein extracts from *L. albus* seeds are able to reduce plasma total cholesterol (Marchesi et al., 2008; Sirtori et al., 2004) and triglyceride concentrations (Sirtori et al., 2004; Spielmann et al., 2007) in animal models. Moreover, it was also shown that a diet containing *L. albus* proteins reduces atherosclerosis progression in rabbits (Marchesi et al., 2008).

A few studies have investigated the potential hypolipidaemic effect of blue lupin (*L. angustifolius*). In pigs fed a diet with whole blue lupin seeds, Martins et al. (2005) reported lower total and low-density lipoprotein (LDL)-cholesterol levels vs. pigs fed a casein-based diet, but no variations in plasma triglyceride levels. In a more recent study in growing rats, comparing diets containing 5% of isolated proteins from different cultivars of blue lupin, no effect on total cholesterol levels was reported and a significant reduction of triglyceridaemia could be observed only for the Vitabor cultivar (Bettzieche, Brandsch, Schmidt et al., 2008). Moreover, apoE-deficient mice fed 10% *L. angustifolius* proteins for 16 weeks displayed higher cholesterol and triglyceride levels vs. casein fed mice (Weisse, Brandsch, Hirche, Eder & Stangl, 2010). On the contrary, a study, performed under peculiar metabolic conditions, i.e. in lactating rats, indicated that a diet containing 20% *L. angustifolius* proteins markedly reduced both cholesterol and triglyceride levels (Bettzieche, Brandsch, Eder, & Stangl, 2009). Altogether, there is no clear indication of a beneficial effect of proteins from *L. angustifolius* in modifying plasma lipid levels. A rat study was therefore designed to evaluate a possible hypolipidaemic effect of blue lupin proteins, following an experimental protocol that previously highlighted hypocholesterolaemic/hypotriglyceridaemic effects of other legume proteins (Rigamonti et al., 2010; Sirtori et al., 2004; Spielmann et al., 2007). It was decided to feed adult male rats with high concentrations (20%) of proteins from *L. angustifolius*. The study demonstrated a strong hypocholesterolaemic effect of blue lupin proteins. Further, the influence of the dietary treatment on major genes involved in cholesterol metabolism was assessed.

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2. Materials and methods

2.1. Lupin protein preparation

Total protein isolate from the Boregine cultivar of *L. angustifolius* seeds was manufactured by the Fraunhofer-Gesellschaft, Fraunhofer Institute (IVV) (Freising, Germany), by an extraction/precipitation process followed by spray drying (D'Agostina et al., 2006). The protein percentage was 91.15 based on dry matter.

2.2. Animals and experimental diets

Procedures involving animals and their care were conducted in accordance with institutional guidelines that are in compliance with national (D.L. No. 116, G.U. Suppl. 40, February 18, 1992, Circolare No. 8, G.U. luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health – NIH Publication No. 85-23, revised 1996). The experiments were supervised by the Laboratory Animal Welfare Service at our Department.

Thirty male Sprague–Dawley rats (Charles River Italia, Calco, Italy; body weight 200–225 g) were housed in a room with controlled lighting (12 h/day), constant temperature and relative humidity. During the first week, they were fed a commercial non-purified diet (Mucedola, Settimo Milanese, Italy) and then divided into two groups of 15 rats on the basis of body weight, so that the distribution between the groups was similar. The rats were then fed for 28 days *ad libitum* a cholesterol/cholic acid diet containing casein as protein source or an identical diet except for the protein content, which was comprised of the total protein isolate from blue lupin. The composition of the semi-synthetic diets is shown in Table 1 and the amino acid concentration of the two diets is reported in Table 2. During the feeding period, body weight and food intake were recorded.

2.3. Sample collection

Fasting blood samples were collected into tubes containing 0.1% (w/v) EDTA before and after 14 and 28 days of dietary treatments. Plasma was separated by centrifugation at 8000 rpm for 10 min at 4 °C and stored at −20 °C for lipid analysis. At the end of the dietary treatments (28 days) rats were sacrificed and liver was excised and immediately snap-frozen in liquid nitrogen for subsequent RNA isolation and analysis.

2.4. Plasma/lipid analyses

Total cholesterol, HDL-cholesterol, and triglyceride plasma concentrations were measured with standard enzymatic techniques, using a Roche Diagnostics Cobas autoanalyser. High-density lipoprotein (HDL)-cholesterol was measured after precipitation of apolipoprotein (apo)B-containing lipoproteins with polyethylene glycol (20%, weight/volume) in 0.2 M glycine (pH 10). This method has been extensively used for the measurement of HDL-cholesterol levels in mice (Chiesa et al., 1998; Parolini et al., 2003; Parolini et al., 2005; Schultz et al., 1992) and has been validated in our laboratory for HDL-cholesterol quantification in rats by comparison with results obtained by fast protein liquid chromatography separation of lipoprotein fractions (data not shown).

Table 1
Composition of experimental diets.

Ingredients (%)	CASEIN	LUPIN
Casein	20	–
Lupin protein isolate	–	20
DL-methionine	0.4	0.4
Hegsted mineral mix	4	4
Coconut oil	25	25
Sucrose	44.1	44.1
Cholesterol	1	1
Cholic acid	0.5	0.5
Vitamins	+	+
Cellulose	5	5

Table 2
Amino acid concentration in diets.

Amino acid (g/kg diet)	CASEIN	LUPIN
Arginine	6.98	23.16
Cystine	0.62	2.90
Glycine	3.22	7.2
Histidine	5.18	4.92
Isoleucine	11.4	8.12
Leucine	17.6	14.12
Lysine	14.28	7.06
Methionine	5.6 + 4.0[a]	0.48 + 4.0[a]
Phenylalanine	9.62	7.4
Serine	10.92	9.12
Tyrosine	9.8	5.88
Threonine	7.82	5.34
Valine	13.4	6.16

[a] Dietary supplementation.

2.5. Real-time PCR analyses

Total RNA was isolated from rat livers using the NucleoSpin RNA extraction kit (Macherey–Nagel, Düren, Germany) according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Total RNA (1 μg) was reverse transcribed with random hexameric primers and MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. cDNAs were quantified by real-time detection polymerase chain reaction (PCR) on an Applied Biosystems 7900 sequence detector using SYBR® Green I and specific primers, as indicated in Table 3. Real-time detection was performed in a volume of 25 μL containing 100 nmol/L of each primer and iTaq SYBR Green Supermix with ROX 2×, as recommended by the manufacturer (Bio-Rad, Hercules, CA). Conditions were 95 °C for 10 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C. A final melting curve guaranteed the authenticity of the target product. The housekeeping gene cyclophilin was used for normalisation. The mRNA concentration of cyclophilin was not influenced by experimental conditions.

2.6. Statistical analysis

Data are expressed as mean values and standard deviations. Group differences were tested for statistical significance by multivariate ANOVA (repeated measures), followed by the Tukey post hoc test; a value of $p < 0.05$ was considered as statistically significant. The statistical analysis was performed using the SYSTAT software (Version 12; Systat Software, Inc., Chicago, IL).

3. Results

3.1. Effect of a lupin protein isolate from L. angustifolius on plasma lipids

The *L. angustifolius* protein isolate did not affect body weight of the rats on the high cholesterol regimen, when monitored during