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RESEARCH ARTICLE

Hypolipidemic effect of dietary pea proteins: Impact on genes regulating hepatic lipid metabolism

Elena Rigamonti, Cinzia Parolini*, Marta Marchesi, Erika Diani, Stefano Brambilla, Cesare R. Sirtori and Giulia Chiesa*

Department of Pharmacological Sciences, Università degli Studi di Milano, Milan, Italy

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Controversial data on the lipid-lowering effect of dietary pea proteins have been provided and the mechanisms behind this effect are not completely understood. The aim of the study was to evaluate a possible hypolipidemic activity of a pea protein isolate and to determine whether pea proteins could affect the hepatic lipid metabolism through regulation of genes involved in cholesterol and fatty acid homeostasis. Rats were fed Nath's hypercholesterolemic diets for 28 days, the protein sources being casein or a pea protein isolate from *Pisum sativum*. After 14 and 28 days of dietary treatment, rats fed pea proteins had markedly lower plasma cholesterol and triglyceride levels than rats fed casein ($p<0.05$). Pea protein-fed rats displayed higher hepatic mRNA levels of LDL receptor *versus* those fed casein ($p<0.05$). Hepatic mRNA concentration of genes involved in fatty acids synthesis, such as fatty acid synthase and stearoyl-CoA desaturase, was lower in pea protein-fed rats than in rats fed casein ($p<0.05$). In conclusion, the present study demonstrates a marked cholesterol and triglyceride-lowering activity of pea proteins in rats. Moreover, pea proteins appear to affect cellular lipid homeostasis by upregulating genes involved in hepatic cholesterol uptake and by downregulating fatty acid synthesis genes.

Keywords:
Cholesterol / Hepatic gene expression / Pea proteins / Rats / Triglycerides

1 Introduction

A major interest for atherosclerosis prevention has been addressed to vegetable proteins, particularly soy proteins whose consumption has been shown to exert hypocholesterolemic [1–3] and hypotriglyceridemic [4, 5] effects in experimental animals as well as in humans. However, a number of drawbacks associated with the use of soybean protein in foods, such as the beany or green flavor and the presence of antinutritional factors, together with the concern about the possible introduction of genetically modified soybeans, has aroused interest in alternative vegetable protein sources. Recently, studies in rats have shown that lupin proteins have a remarkable efficacy in reducing both plasma cholesterol and triglyceride levels [6, 7], thus confirming the hypolipidemic potential of legume proteins.

Another protein-rich legume is pea, whose seeds contain about 25% of crude protein [8]. Although peas are widely used in animal nutrition, human consumption of peas is lower than that of other traditionally more accepted pulses [9]. Nevertheless, the wealth of nutrients available from the pea and their beneficial functional properties have prompted increasing interest and demand for this legume for food preparations addressed to geriatric and infant nutrition [10]. In a recent clinical study, pea proteins displayed a better satiating effect in respect to animal proteins, thus indicating a new potential use of this food component for human health [11].

Correspondence: Dr. Giulia Chiesa, Department of Pharmacological Sciences, Università degli Studi di Milano, Milan, Italy
E-mail: giulia.chiesa@unimi.it
Fax: +39-02-50318284

Abbreviations: apo, apolipoprotein; **CYP7A1,** cholesterol 7α-hydroxylase; **FAS,** fatty acid synthase; **HMG-CoA,** hydroxymethyl-glutaryl-CoA; **SCD,** stearoyl-CoA desaturase; **SREBP,** sterol regulatory element-binding protein

*These authors contributed equally to this work

Early studies on the impact of pea proteins on plasma lipids demonstrated a marked hypolipidemic activity of this dietary component [12, 13], whereas no effects were shown in a more recent investigation [14].

The aim of the present study was to evaluate, in an animal model, the hypolipidemic properties of pea proteins and to investigate whether they could affect liver metabolism by regulating genes involved in cholesterol and fatty acid homeostasis. The study was performed on rats maintained for 28 days on a hypercholesterolemic diet containing casein or a pea protein isolate as protein source. A marked hypocholesterolemic and hypotriglyceridemic effect of the pea protein isolate was observed. To investigate a possible impact of pea proteins on the expression of genes involved in cholesterol metabolism, the relative mRNA concentration of sterol regulatory element-binding protein (SREBP)-2 and that of its target genes such as hydroxymethyl-glutaryl-CoA (HMG-CoA) reductase and LDL receptor was determined, together with the hepatic gene expression of cholesterol 7α-hydroxylase (CYP7A1). To highlight possible mechanisms by which pea proteins may exert their hypotriglyceridemic effect, the hepatic gene expression of SREBP-1c and its target genes involved in fatty acid biosynthesis, such as glucose-6-phosphate dehydrogenase, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)-1, -2, and acyl-CoA:glycerol-3-phosphate acyltransferase was evaluated.

2 Materials and methods

2.1 Animals and experimental diets

Twenty-four male Sprague–Dawley rats (Charles River Italia) weighing 200–225 g were fed a Nath's hypercholesterolemic diet for 1 week (20% casein, 1% cholesterol and 0.5% cholic acid), and then randomly divided into two groups of 12 animals each on the basis of total cholesterolemia and body weight. Rats were then fed *ad libitum* a Nath's hypercholesterolemic diet [15] for 28 days, the protein source being either total protein isolate from *Pisum sativum* (Pisane® C9; Provital Industrie S.A. Belgium), or casein as the control protein. Pisane® C9 was constituted by 90.8% pea proteins on dry matter and low amounts of fat (1.2%) and fiber (0.9%). The composition of the semi-synthetic diets is shown in Table 1 and the amino acid composition is shown in Table 2. The Nath's diet has, as exclusive fat source, coconut oil, a dietary fat with a low content of linoleic and α-linolenic acid [16]. However, due to the relatively short duration of treatment, the occurrence of an essential fatty acid deficiency could be excluded [16].

During the feeding period, body weight and food intake were recorded. All rats were housed in a room with controlled temperature (18°C), relative humidity (55–65%) and a 12 h–12 h light–dark cycle. All experimental procedures involving animals and their care were conducted in compliance with national and European Union laws and policies.

Table 1. Composition of experimental diets

Ingredients (%)	Casein	Pea
Casein	20	–
Pea 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	Pea
Arginine	6.98	16.38
Histidine	5.18	4.86
Isoleucine	11.4	8.46
Leucine	17.6	15.66
Lysine	14.28	13.86
Methionine	5.6+4.0[a)]	2.16+4.0[a)]
Cystine	0.62	1.98
Phenylalanine	9.62	10.08
Tyrosine	9.8	7.02
Threonine	7.82	7.02
Tryptophan	2.16	1.8
Valine	13.4	9.36
Glycine	3.22	7.38
Serine	10.92	10.08

a) Dietary supplementation.

2.2 Sample collection

Fasting blood samples were collected from the retroorbital plexus 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 the liver was excised and immediately snap-frozen in liquid nitrogen for subsequent RNA isolation and analysis.

2.3 Plasma lipid analysis

Plasma total cholesterol, HDL-cholesterol, and triglyceride concentrations were measured with standard enzymatic techniques by using a Roche Diagnostics Cobas auto-analyser. HDL cholesterol was measured after precipitation of apolipoprotein (apo)B-containing lipoproteins with polyethylene glycol (20%, w/v) in 0.2 mol/L glycine (pH 10). This method has been extensively used for the measurement of HDL-cholesterol levels in mice [17–20] and has been validated in our laboratory for HDL-cholesterol quantification in

rats by comparison with results obtained by fast protein LC separation of lipoprotein fractions (data not shown).

2.4 Real-time PCR analysis

Total RNA was isolated from rat livers using the NucleoSpin RNA extraction kit (Macherey-Nagel) 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) following the manufacturer's instructions. cDNAs were quantified by real-time detection PCR on an Applied Biosystems 7900 sequence detector using SYBR® Green I and specific primers 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). 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 normalization. The mRNA concentration of cyclophilin was not influenced by experimental conditions.

2.5 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 statistically significant. The statistical analysis was performed using the SYSTAT software (version 5.2; Systat Software).

3 Results

3.1 Effect of a total protein isolate from *Pisum sativum* on plasma lipids

At the end of the dietary treatment, weight gains were not different between the two groups of rats (415.45 ± 33.63 g in casein *versus* 432.00 ± 49.89 g in pea protein-fed rats, $p = 0.237$). Plasma total cholesterol concentrations in rats fed pea protein isolate was significantly decreased in comparison with casein-fed animals after both 14 and 28 days of dietary treatment ($p<0.001$) (Fig. 1A). In rats fed the pea protein isolate there was a tendency for HDL cholesterol to be higher than in casein-fed animals, but no statistical significance was achieved (Fig. 1B). Moreover, the pea protein-containing diet determined lower triglyceride levels compared with casein at both 14 and 28 days of dietary treatment ($p<0.05$) (Fig. 1C).

3.2 Effect on hepatic mRNA concentration of genes involved in cholesterol metabolism

In order to examine possible mechanisms for the pea protein-mediated alterations of cholesterolemia, liver mRNA concentrations of genes involved in cholesterol synthesis, cholesterol uptake, and bile acid synthesis were measured by real-time PCR. Relative mRNA concentrations of SREBP-2 and HMG-CoA reductase were not influenced by the dietary treatment (Fig. 2). Moreover, the initial and rate-limiting enzyme of bile acid synthesis, CYP7A1, was also not influenced at the transcriptional level by pea proteins (Fig. 2). Remarkably, pea proteins compared with casein significantly increased mRNA levels of the LDL receptor, the major regulator of circulating LDL cholesterol (Fig. 2).

Table 3. Sequences of the primers used for real-time PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)
ApoB	CGGTGGCAGAAATAACGTCT	GGGCTCACATTATTGGCTGT
Cyclophilin	AGCACTGGGGAGAAAGGATT	AGCCACTCAGTCTTGGCAGT
CYP7A1	CACCATTCCTGCAACCTTTT	GTACCGGCAGGTCATTCAGT
FAS	TCGAGACACATCGTTTGAGC	TCAAAAAGTGCATCCAGCAG
GPAT	CAGCGTGATTGCTACCTGAA	CTCTCCGTCCTGGTGAGAAG
G6PDH	AGCCTCCTACAAGCACCTCA	TGGTTCGACAGTTGATTGGA
HL	TGCCAATTTTGTGGATGCTA	TTAAGCCATGCTCTGCAATG
HMG-CoA reductase	CCCAGCCTACAAACTGGAAA	CCATTGGCACCTGGTACTCT
LDL receptor	CAGCTCTGTGTGAACCTGGA	TTCTTCAGGTTCGGGATCAG
MTTP	AGACTCCAGCCTCACTGGAA	TGCAGCCTTCATTCTGACAC
SCD1	GATATCCACGACCCCAGCTA	CCCAGGGCACTGATAAGGTA
SCD2	CCAGAGCGTACCAGCTTTTC	TTACCCACTTCGCAAGCTCT
SREBP-1c	GGAGCCATGGATTGCACATT	AGGAAGGCTTCCAGAGAGGA
SREBP-2	AGACTTGGTCATGGGGACAG	GGGGAGACATCAGAAGGACA

ApoB, apolipoprotein (apo) B; CYP7A1, cholesterol 7α-hydroxylase; FAS, fatty acid synthase; GPAT, acyl-CoA:glycerol-3-phosphate acyltransferase; G6PDH, glucose-6-phosphate dehydrogenase; HL, hepatic lipase; HMG-CoA, hydroxymethyl-glutaryl-CoA; MTTP, microsomal triglyceride transfer protein; SCD, stearoyl-CoA desaturase; SREBP, sterol regulatory element-binding protein.

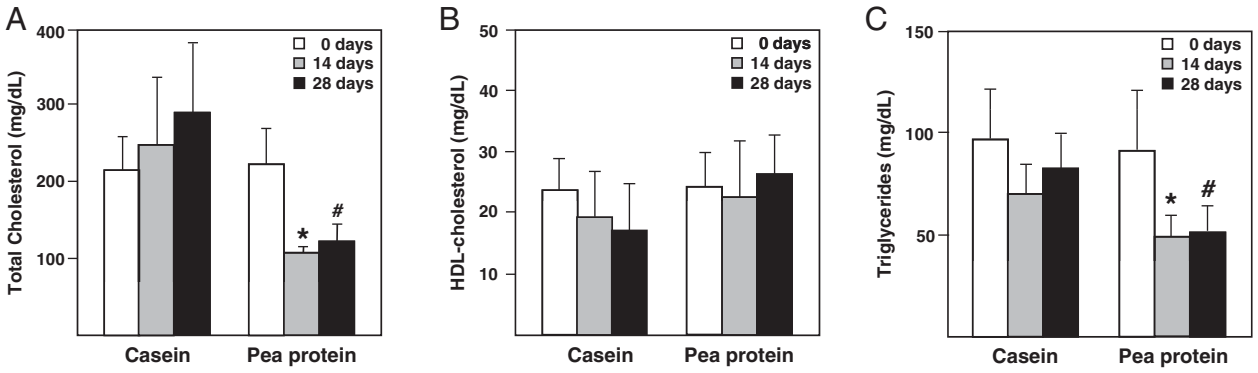

Figure 1. Plasma concentrations of total cholesterol (A), HDL cholesterol (B) and triglycerides (C) in male rats fed diets containing casein or pea protein for 28 days. Plasma lipid analysis was performed before, during (14 days) and at the end of dietary treatment (28 days). Each bar represents mean values ± SD ($n = 12$). Statistically significant differences between dietary treatments are indicated (pea protein *versus* casein-fed group at 14 and 28 days: $^{*}p < 0.05$, $^{\sharp}p < 0.05$).

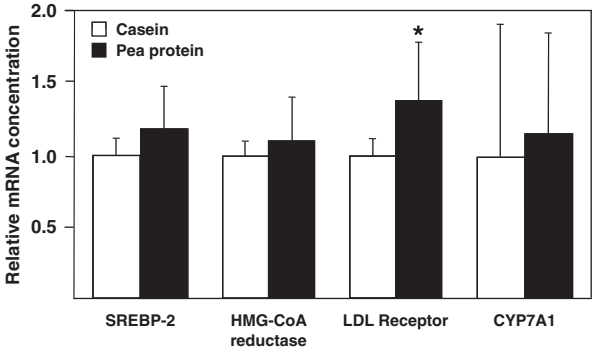

Figure 2. Relative mRNA concentrations of SREBP-2, HMG-CoA reductase, LDL receptor and CYP7A1 in the liver of male rats fed diets containing casein or pea protein for 28 days. Values were normalized to reference gene cyclophilin and are expressed relative to the levels in casein-fed animals set as 1. Each bar represents mean values ± SD ($n = 12$). Statistically significant differences between dietary treatments are indicated (pea protein *versus* casein-fed group: $^{*}p < 0.05$).

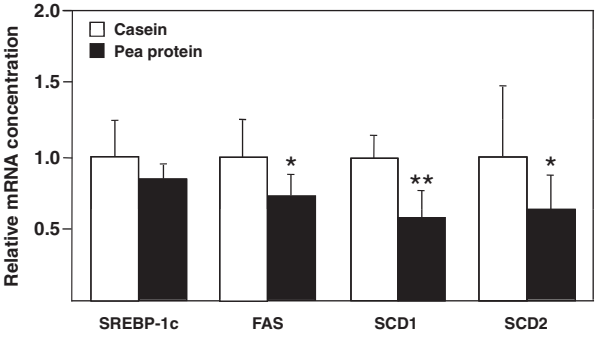

Figure 3. Relative mRNA concentrations of SREBP-1c, FAS, SCD1 and SCD2 in the liver of male rats fed diets containing casein or pea protein for 28 days. Values were normalized to reference gene cyclophilin and are expressed relative to the levels in casein-fed animals set as 1. Each bar represents mean values ± SD ($n = 12$). Statistically significant differences between dietary treatments are indicated (pea protein *versus* casein-fed group: $^{*}p < 0.05$; $^{**}p < 0.001$).

3.3 Effect on hepatic mRNA concentration of genes involved in triglyceride metabolism

To gain further insights into the lipid-modulating effects of pea proteins, the expression of genes involved in triglyceride homeostasis was investigated. The relative concentration of the transcription factor SREBP-1c in the liver tended to be lower in the group fed pea proteins than in the group fed casein, although no statistical significance was achieved. Interestingly, relative mRNA concentration of SREBP-1c target genes was influenced by the dietary treatment. Particularly, hepatic mRNA concentrations of FAS, SCD1 and SCD2 were downregulated in rats fed pea proteins compared with rats fed casein (Fig. 3). Relative mRNA concentrations of genes involved in apoB-containing lipoprotein turnover, such as apoB, microsomal triglyceride transfer protein and hepatic lipase were also assessed, but they were unaffected by the type of diet (data not shown).

4 Discussion

In the present study, a diet containing an isolate from *Pisum sativum* as protein source displayed marked hypolipidemic properties in rats. To our knowledge, all the animal studies investigating the impact of whole pea seeds on plasma lipids have shown a hypocholesterolemic effect of this dietary approach [21–25], whereas the impact on triglyceride plasma levels has remained unclear [21, 22, 24, 25].

Very few studies have tried to identify the pea seed components responsible for the observed hypolipidemic activity. Pea fibers seem to exert some beneficial effects, as

shown in two human studies on normolipidemic subjects [26, 27]. The protein fraction of pea seeds could also display hypolipidemic properties, similar to other legume proteins [6, 28]. Early investigations on pea proteins, demonstrated marked hypocholesterolemic [12, 13] and hypotriglyceridemic [13] effects of this dietary component compared with casein as the protein source. In contrast, a very recent study on rats fed a diet containing pea proteins failed to show any difference in plasma cholesterol and triglycerides compared with casein-fed animals [14]. The described lack of hypolipidemic activity could be due to ethanol washing of the pea protein isolate [14], introduced by the authors to remove isoflavones from the preparation. This procedure might have altered the biological activity of the protein isolate, as previously suggested for other protein preparations [29] and well documented by proteomic analysis of a variety of soy protein products [30]. It should be noted that peas are characterized by extremely low levels of isoflavones (more than 1000 times below those found in soy) [31], therefore the concentration of these molecules in pea protein preparations can be considered too low to exert any significant biological activity.

Indeed, the results of the present study confirm a marked hypocholesterolemic and hypotriglyceridemic effect of pea proteins in rats and imply a potential benefit of pea proteins on human health.

Previous studies have indicated that the amino acid composition of dietary proteins may influence plasma lipid levels [32]. Particularly, low methionine concentrations and elevated arginine:lysine ratio have been implicated in the hypolipidemic properties of vegetable proteins [33]. Pea proteins have a lower methionine concentration compared with casein and, since the two diets were equally supplemented for methionine (4 g/kg diet), the two dietary treatments were not balanced for the methionine content (9.6 g/kg in the casein diet *versus* 6.16 g/kg in the pea protein diet, see Table 2). This difference, however, should not have a major impact on plasma lipid levels, as indicated in a recent study [34] where it was demonstrated that above a concentration of 3.5 g/kg diet, the methionine content does not influence plasma cholesterol levels. As in all legume proteins, pea proteins are characterized by an elevated arginine:lysine ratio compared with casein. In the present study, the arginine:lysine ratio was 1.18 in the pea-containing diet *versus* 0.49 in the casein-containing diet. On the basis of previous studies [33, 35], it cannot be excluded that this difference in amino acid composition between the two diets may have contributed to the observed hypolipidemic effect of pea proteins. However, a very recent study in rats maintained at dietary treatments differing only for arginine and lysine concentrations did not support the hypothesis that an elevated dietary arginine:lysine ratio influence plasma lipid levels [36].

Finally, specific peptides may be responsible for the observed effects. Very little is known on bioactive legume peptides except for soy, where several peptides with hypolipidemic activity have been identified [37, 38]. Owing to the homology among legume proteins, as recently demonstrated for soy and lupin [39], it is not unlikely that different legumes may exert their hypolipidemic effect through homologous peptides.

A major focus of the present study was the investigation of potential mechanisms explaining the impact of pea proteins on circulating plasma total cholesterol and triglycerides. In order to examine the hypocholesterolemic effect of pea proteins, hepatic mRNA concentrations of SREBP-2, its target genes HMG-CoA reductase and LDL receptor, as well as CYP7A1 were measured. Whereas no relevant variations of SREBP-2, HMG-CoA reductase and CYP7A1 were observed, the LDL-receptor expression was significantly elevated in pea protein-fed animals compared with controls. The LDL receptor is a major regulator of circulating LDL-cholesterol levels [40], and increased hepatic LDL-receptor expression results in accelerated clearance of LDL particles [37]. The observed elevation of hepatic LDL-receptor mRNA concentration in pea protein-fed animals may therefore result in an increased LDL catabolism and contribute to the observed plasma cholesterol reduction in these animals. Previous studies have demonstrated that proteins purified from soy or white lupin seeds exert an hypocholesterolemic action through a stimulation of LDL-receptor activity [6, 37]. Altogether, these data suggest that legume proteins may exert their hypocholesterolemic effect through similar mechanisms and possibly through common protein/peptide sequences.

To investigate potential mechanisms explaining the hypotriglyceridemic effect of pea proteins, mRNA transcript levels of genes involved in the *de novo* synthesis of fatty acids, as well as of genes associated with triglyceride hepatic secretion and hydrolysis were measured in livers of casein and pea protein-fed animals. The pea protein-based diet did not influence triglyceride secretion or hydrolysis compared with the casein diet, whereas an effect was observed on fatty acid synthesis. SREBP-1c is a key regulator of fatty acid and triglyceride synthesis in the liver [41]. An increase of the nuclear concentration of SREBP-1c, occurring through an increased gene expression or enhanced proteolytic activation, leads to transcription activation of genes encoding fatty acid synthesis enzymes [41, 42]. In the present study, a modest, not significant reduction of SREBP-1c gene expression was observed in pea protein-fed rats compared with casein-fed animals, whereas mRNA concentrations of SREBP-1c target genes (FAS, SCD1, SCD2) were markedly lower in pea protein-fed animals. The significant downregulation of FAS, SCD1 and SCD2 in spite of an almost absent reduction of SREBP-1c expression could be explained by a reduced proteolytic activation of SREBP-1c and a consequent reduced nuclear concentration of the activated transcription factor [43]. Altogether, the present results clearly indicate that pea proteins exert a hypotriglyceridemic activity mainly through downregulation of fatty acid synthesis. Results from other authors suggest a similar

mechanistic explanation for the hypotriglyceridemic effect exerted by lupin proteins [7]. These observations again suggest that common pathways may explain the hypolipidemic effect of legume proteins.

In conclusion, this study demonstrates a marked hypocholesterolemic and hypotriglyceridemic effect of a pea protein-based diet in hypercholesterolemic rats and suggests that these effects may occur, respectively, through upregulation of LDL receptor and downregulation of fatty acid synthesis.

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