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Research Article

Lupin protein acts hypocholesterolemic and increases milk fat content in lactating rats by influencing the expression of genes involved in cholesterol homeostasis and triglyceride synthesis

Anja Bettzieche, Corinna Brandsch, Klaus Eder and Gabriele I. Stangl

Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Halle (Saale), Germany

Lupin protein has been shown to reduce triglyceride and cholesterol concentrations in plasma of hypercholesterolemic growing and adult rats. However, the effect of lupin protein on lipid metabolism during pregnancy and lactation is unknown. Female rats were assigned to two groups and were fed a hypercholesterolemic diet containing either 200 g/kg lupin protein or casein during pregnancy until day 18 of lactation. Dams fed lupin protein had lower triglyceride concentrations in plasma (−55%) and higher triglyceride concentrations in liver (>2-fold) and milk (+81%) than dams fed casein ($p < 0.05$). The concentration of cholesterol in plasma, VLDL, LDL, and liver was markedly lower and the concentration of HDL cholesterol was higher in rats fed lupin protein than in rats fed casein ($p < 0.05$). Lupin protein induced a 2.6-fold increase of hepatic LDL receptor concentration compared to casein ($p < 0.05$), down-regulated genes involved in fatty acid oxidation in the liver, and up-regulated lipogenic genes in the mammary gland ($p < 0.05$). This study shows that lupin protein increases milk fat content and strongly modifies triglyceride and cholesterol metabolism by influencing the transcription levels of genes involved in fatty acid oxidation and synthesis and cholesterol homeostasis.

Keywords: Gene expression / Lactating rats / Lupin protein / Milk / Plasma and liver lipids

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1 Introduction

Sweet varieties of lupin have been cultivated for centuries for domestic animal feed, but also for human nutrition, mainly in several parts of Australia, Europe, and South America. Their use in the food industry is being developed, and lupin protein is beginning to replace soybean protein in several food products [1]. Recent studies have shown that protein extracts from lupin seed are also capable of lowering plasma cholesterol [2, 3] and triglyceride concentrations [4, 5] in hypercholesterolemic laboratory animals.

The recently published effects of lupin protein on circulating lipids and lipid metabolism were obtained exclusively from growing and adult rats and rabbits. During pregnancy and lactation, the regulation of lipid metabolism is completely different from growth or maintenance. Firstly, maternal hypertriglyceridemia is a characteristic feature during pregnancy and corresponds to an accumulation of triglycerides not only in VLDL but also in LDL and HDL [6]. Secondly, during late gestation lipoprotein lipase (LPL) activity decreases in the liver and increases in the mammary gland tissue [7]. This shift plays an important role in the fate of circulating triglycerides which are diverted from uptake by the liver to uptake by the mammary gland for milk synthesis. Normally, during lactation, the utilization of metabolic substrates such as fatty acids for the synthesis of milk in the mammary gland is increased and the utilization of substrates for oxidation in other tissues such as liver and skeletal muscle is reduced [8]. The synthesis of milk trigly-

Correspondence: Professor Gabriele I. Stangl, Institut für Agrar und Ernährungswissenschaften, Martin-Luther-Universität Halle-Wittenberg, Von-Danckelmann-Platz 2, D-06120 Halle (Saale), Germany
E-mail: gabriele.stangl@landw.uni-halle.de
Fax: +49-345-5527124

Abbreviations: ACO, acyl coenzyme A oxidase; **apo,** apolipoprotein; **CPT,** carnitine palmitoyl transferase; **CYP7A1,** cholesterol 7α-hydroxylase; **FABP,** fatty acid binding protein; **FAS,** fatty acid synthase; **FAT,** fatty acid translocase; **FATP,** fatty acid transport protein; **HL,** hepatic lipase; **HMG-CoA,** 3-hydroxy-3-methylglutaryl coenzyme A; **LPL,** lipoprotein lipase; **NEFA,** nonesterified fatty acids; **PPAR,** peroxisome proliferator-activated receptor; **SCD,** stearoyl coenzyme A desaturase; **SREBP,** sterol regulatory element-binding protein

cerides depends on the availability of fatty acids in the mammary gland which derive from two different sources. The first source represents the *de novo* biosynthesis of fatty acids in the mammary gland cells by the activity of lipogenic enzymes which is controlled by the lipogenic transcription factor sterol regulatory element-binding protein (SREBP)-1c [9]. The other important source for milk triglyceride synthesis are lipoprotein-bound fatty acids from plasma or nonesterified fatty acids (NEFA) which are released from the adipose tissue and taken up into the mammary gland [10]. Any alteration of these metabolic pathways could influence milk fat synthesis.

Due to the fact that lupin protein is widely used in animal and human nutrition, we consider it necessary to test the effect of lupin protein compared to casein on the milk fat content and the lipid metabolism during lactation. The rat has been suggested to be a suitable model because rats, like humans, develop similar changes in lipid metabolism during pregnancy and lactation [11]. Data from recent studies with adult and growing rats have demonstrated that lupin protein compared to casein downregulates genes involved in lipogenesis [4, 5] and upregulates genes involved in triglyceride hydrolysis [4]. The cholesterol-lowering effect of lupin protein seems to be caused by an increased uptake of cholesterol into cells *via* the LDL receptor [2]. Recent data indicate that the hypolipemic effects of lupin protein are probably caused by alterations of the activities of SREBPs in the liver [4, 5]. SREBPs directly activate the expression of more than 30 genes involved in the regulation of cellular lipid concentrations [12 – 15]. While SREBP-2 is primarily responsible for the regulation of genes involved in cholesterol biosynthesis and uptake of cholesterol in cells [16 – 18], SREBP-1c is mainly involved in the regulation of genes involved in fatty acid biosynthesis [19].

In this study, we aimed to investigate for the first time whether lupin protein also influences the lipid metabolism of lactating rats and the triglyceride content of milk by influencing SREBPs and the mRNA expression of other genes such as the peroxisome proliferator-activated receptor (PPAR)-α which is involved in the fatty acid catabolism or cholesterol 7α-hydroxylase (CYP7A1), a key enzyme for the bile acid synthesis. Since lupin protein exerts stronger effects on lipid metabolism in rats receiving a hypercholesterolemic diet than in rats receiving a normocholesterolemic diet [4], all dams in this study were fed a hypercholesterolemic diet to receive maximum effects of the dietary protein.

2 Materials and methods

2.1 Animals

Twenty-four 9-wk-old female Sprague – Dawley rats (Charles River, Sulzfeld, Germany) with a body weight of 235 ± 10 g (means ± SD) were randomly assigned to 2 groups of 12 rats each. The rats were kept individually in Macrolon cages in a room maintained with controlled temperature (22 ± 2°C), humidity (50 – 60%), and lighting from 06.00 to 18.00 h and consumed a commercial standard rodent diet ("altromin 1324," Altromin, Lage, Germany) *ad libitum*. At 16 wk of age, the rats were mated by housing one male rat with two female rats which received the experimental diets with casein (control diet) or lupin protein. At the day of parturition, designated as day 1 of lactation, litters were adjusted to 10 pups/dam and dams were fed the experimental diets until day 18 of lactation. All experimental procedures described followed established guidelines for the care and handling of laboratory animals and were approved by the council of Saxony-Anhalt, Germany.

2.2 Diets and feeding

All rats were fed a semisynthetic diet, composed according to the recommendations of the American Institute of Nutrition for rats during reproduction [20]. For inducing hypercholesterolemia, the diet was supplemented with 10 g/kg cholesterol and 5 g/kg sodium choleate at the expense of corn starch. Accordingly, such diets have been used to study the effects of nutrients on the lipid metabolism in hypercholesterolemic rats [2, 4, 21 – 24]. The experimental proteins were added in an amount of 200 g/kg. The group fed casein served as a control. Since the concentrations of the indispensable amino acids methionine and lysine were below the recommendations of the NRC for pregnant and lactating rats [25], the casein diet was supplemented with methionine and the lupin protein diet was supplemented with methionine and lysine to meet the requirements. Vitamins and minerals were supplemented according to recommendations of the American Institute of Nutrition for rat diets [20]. Fat was added in an amount of 95 g lard and 5 g linseed oil *per* kg diet to meet the requirements for essential fatty acids. The composition of the semisynthetic diets is shown in Table 1. During the experimental period, the animals had free access to food and water. The experimental diets were fed during mating, pregnancy until day 18 of lactation.

2.3 Preparation and characterization of the dietary proteins

Casein was obtained from Meggle (Wasserburg, Germany) and was not further processed. The lupin protein isolate was extracted from deoiled blue lupin (*Lupinus angustifolius*) and was available from the Fraunhofer Institute (IVV, Freising, Germany). It contained 16 mg alkaloids *per* kg protein isolate. Lupanine was the most abundant quinolizidine alkaloid, followed by 13α-hydroxylupanine and angustifoline.

Concentrations of the isoflavones genistein, daidzein, and genistin in the lupin protein were analyzed by an HPLC

Table 1. Composition of the experimental diets (g/kg)

	Casein diet	Lupin protein diet
Casein	200	–
Lupin protein	–	200
Corn starch	390	390
Sucrose	206	203
Lard	95	95
Linseed oil	5	5
Cellulose	35	35
Vitamin and mineral mixture	50	50
Cholesterol	10	10
Sodium choleate	5	5
DL-methionine	4	6
L-lysine	–	1

method [26]. In brief, lupin protein was extracted with a mixture of methanol and dichlormethane (1:1 v/v). After evaporation of the organic solvents, isoflavones were dissolved in ethanol and used for a quantitative HPLC analysis of genistein, daidzein, and genistin using an ELITE LaChrom HPLC system (Hitachi, Mannheim, Germany) with a gradient of ACN and deionized water including 0.1% trifluoracetic acid as mobile phase at a flow rate of 0.8 mL/min. Isoflavones were detected at 260 nm. The concentrations of genistein, daidzein, and genistin of the lupin protein used in this study were below the detection limits of 2.22 µmol/kg for genistein, 1.18 µmol/kg for daidzein, and 1.39 µmol/kg for genistin.

For analysis of the amino acid concentrations in the diet, samples were oxidized and then hydrolyzed with 6 M HCl [27]. Separation and quantification of the amino acids were performed by ion exchange chromatography following postcolumn derivatization in an amino acid analyzer (Biotronic LC 3000, Eppendorf, Hamburg, Germany) [28]. Tryptophan was determined by digesting the diet with barium hydroxide [29]. The dietary tryptophan concentration was detected by RP HPLC [30]. The concentrations of amino acids in the diets containing the lupin proteins and the casein are shown in Table 2.

2.4 Sample collection

At day 15 of lactation, milk samples were collected from the dams. After separation from the pups for 1 h, dams were anesthetized i.m. with ketamine (75 mg/kg body weight) and injected i.m. with 1 IU oxytocin to stimulate milk flow. Milking was performed at 10.00 a.m. with a milking machine. From each rat, 2–3 mL of milk were obtained from all teats within 10 min through below atmospheric pressure. Samples were stored at −20°C until analysis. At day 18 of lactation the dams and pups were killed by decapitation under light anesthesia with diethyl ether. The last portion of food was given 4 h before killing as food deprivation leads to a significant downregulation of the genes involved in lipid metabolism which were considered in this study [17, 19]. Whole blood was collected into heparinized polyethylene tubes and plasma was separated by centrifugation at $1100 \times g$ for 10 min at 4°C. Plasma samples were stored at −20°C. Livers and mammary glands were excised, weighed, and immediately snap frozen in liquid nitrogen and stored at −80°C.

Table 2. Amino acid composition of the experimental diets (g/kg)

	Casein diet	Lupin protein diet
Alanine	5.5	6.0
Arginine	6.3	22.6
Aspartic acid	13.2	20.6
Cysteine	0.8	2.7
Glutamic acid	42.2	46.4
Glycine	3.4	7.8
Histidine	6.4	5.2
Isoleucine	8.8	8.8
Leucine	17.7	14.4
Lysine	14.7	9.3
Methionine	10.1	9.5
Phenylalanine	9.7	8.2
Proline	20.2	8.4
Serine	10.8	9.3
Threonine	7.9	6.3
Tryptophan	1.9	1.6
Tyrosine	8.6	6.2
Valine	12.1	7.4

2.5 Lipid analysis

Plasma lipoproteins chylomicrons, VLDL, LDL, and HDL were separated by step-wise ultracentrifugation ($900\,000 \times g$ at 4°C for 1.5 h; Mikro-Ultrazentrifuge, Sorvall Products, Bad Homburg, Germany) by appropriate density cuts (chylomicrons, $\rho < 0.95$ kg/L; VLDL, $0.95 < \rho < 1.006$ kg/L; LDL, 1.006 kg/L $< \rho < 1.040$ kg/L; HDL, $\rho > 1.063$ kg/L). Each density fraction was separated by SDS-PAGE and proven to contain the apolipoproteins (apo) typical for each type of lipoprotein. Plasma densities were adjusted by sodium chloride and potassium bromide. For the measurement of liver and milk triglycerides and of liver cholesterol, lipids were extracted with a mixture of *n*-hexane and isopropanol (3:2 v/v) [31]. The concentrations of lipids in liver were determined using an enzymatic reagent kit after drying an aliquot of the lipid extracts and dissolving the lipids with Triton X-100 [32]. Concentrations of cholesterol and triglycerides in plasma and lipoproteins, liver and milk were examined using enzymatic reagent kits (DiaSys Diagnostic Systems, Holzheim, Germany, Cat. No. 1.1300 99 90 314 and 1.5760 99 90 314). The concentration of NEFA in plasma was measured by using an enzymatic reagent kit (Wako Chemicals, Neuss, Germany).

Table 3. Sequences and annealing temperatures of the primers used for real-time RT-PCR analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	bp	Temperature (°C)	Accession number
ACO	CTTTCTTGCTTGCCTTCCTTCTCC	GCCGTTTCACCGCCTCGTA	415	60	NM 017340
ApoA1	CCTGGATGAATTCCAGGAGA	TCGCTGTAGAGCCCAAACTT	192	60	NM_012738
ß-Actin	ATCGTGCGTGACATTAAAGAGAAG	GGACAGTGAGGCCAGGATAGAG	429	60	NM 031144
CPT-1a	GGAGACAGACACCATCCAACATA	AGGTGATGGACTTGTCAAACC	416	60	NM 031559
CYP7A1	CAAGACGCACCTCGCTATCC	CCGGCAGGTCATTCAGTTG	206	60	NM 012942
FABP	ACCATCCACTGCCGTCTTAC	CCCCGATGCGTAGGTATTCT	185	60	NM 012556
FAS	AGGTGCTAGAGGCCCTGCTA	GTGCACAGACACCTTCCCAT	281	60	NM 017332
FAT/CD36	TCGTATGGTGTGCTGGACAT	GGCCCAGGAGCTTTATTTTC	194	60	NM 031561
FATP	GGTAGCAAATGCACCCTCAT	CTCCTGCTGTGATGTGAGGA	235	60	NM 053580
HL	TCCCACCACAAGTACA	TCAGCCAGGGCATTATTTTC	167	60	BC 088160
HMG-CoA reductase	AAGGGGCGTGCAAAGACAATC	ATACGGCACGGAAAGAACCATAGT	406	57	NM 013134
LDL-receptor	AGAACTGCGGGGCCGAAGACAC	AAACCGCTGGGACATAGGCACTCA	490	65	NM 175762
LPL	TCCCACCACAACGAAGTACA	TCAGCCAGGGCATTATTTTC	205	60	NM 012598
PPAR-α	CCCTCTCTCCAGCTTCCAGCCC	CCACAAGCGTCTTCTCAGCCATG	177	60	NM 013196
SCD-1	CCAGAGCGTACCAGCTTTTC	TTACCCACTTCGCAAGCTCT	195	60	NM 139192
SREBP-1c	GGAGCCATGGATTGCACATT	AGGAAGGCTTCCAGAGAGGA	191	60	XM 001075680
SREBP-2	ATCCGCCCACACTCACGCTCCTC	GGCCGCATCCCTCGCACTG	214	65	XM 216989

ACO, acyl coenzyme A oxidase; Apo, apolipoprotein; CPT, carnitine palmitoyl transferase; CYP7A1, cholesterol 7α-hydroxylase; FABP, fatty acid binding protein; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; HL, hepatic lipase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; SCD, stearoyl coenzyme A desaturase; SREBP, sterol regulatory element-binding protein.

2.6 Western blot analysis of LDL receptor

LDL receptor protein abundance in the liver tissue was measured by Western blot analysis according to methods of Lemieux *et al.* [33] and Marino *et al.* [34]. To extract the LDL receptor protein, ~200 mg liver sample was homogenized in 1 mL buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholeate, 1% v/v Triton X-100, 0.1% v/v SDS, 10% v/v glycerol, and 1 mM EDTA. The crude extract was centrifuged at 13 000 × *g* for 10 min at 4°C, and the supernatant was removed and stored at –80°C until further processing. The protein content of the samples was determined by the bicinchoninic acid assay. Ten micrograms of protein per lane were separated on a 10% SDS-polyacrylamide gel according to the method of Laemmli [35] and electrotransferred to a nitrocellulose membrane (Bio Trace NT, Pall Corporation, Pensacola, FL, USA). The membrane was incubated overnight with 1:200 polyclonal antirabbit LDL receptor (Abcam, Cambridge, UK), and then with 1:5000 horseradish peroxidase-linked antirabbit IgG (Sigma–Aldrich, Taufkirchen, Germany). Bands were detected using the chemiluminescent agent ECL Plus Western Blotting Detection Reagents (Amersham Biosciences Europe, Freiburg, Germany) and a chemiluminescence imager camera (F-ChemisBIS, Biostep, Jahnsdorf, Germany).

2.7 RT-PCR analysis

Total RNA was isolated from rat livers with TRIZOL™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the OD at 260 and 280 nm, respectively. Total RNA (1.2 µg) was subjected to first strand cDNA synthesis at 42°C for 60 min using M-MuLV reverse transcriptase (MBI Fermentas, St. Leon-Rot, Germany), and oligo dT18-primer (Operon Biotechnologies, Cologne, Germany). The relative mRNA quantities of acyl coenzyme A oxidase (ACO) (EC 1.3.3.6), β-actin, apoA1, carnitine palmitoyl transferase (CPT)-1a (EC 2.3.1.2.1), CYP7A1 (EC 1.14.13.17), fatty acid binding protein (FABP), fatty acid translocase (FAT)/CD36, fatty acid transport protein (FATP), fatty acid synthase (FAS) (EC 2.3.1.65), hepatic lipase (HL) (3.1.1.3), 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), LDL receptor, LPL (EC 3.1.1.34), PPAR-α, stearoyl coenzyme A desaturase (SCD)-1 (EC 1.14.19.1), SREBP-1c and SREBP-2 were measured by real-time detection PCR using SYBR® Green I (Sigma) and the Rotor Gene 2000 system (Corbett Research, Mortlake, Australia). Real-time detection PCR was performed with 1.25 U Taq DNA polymerase (Promega, Mannheim, Germany), 500 µM dNTPs and 26.7 pmol of the specific primers (Operon Biotechnologies). Sequences and annealing temperatures of the primers used for real-time PCR are presented in Table 3. For determination of mRNA concentration a threshold cycle (C_t) and amplification efficiency was obtained from each amplification curve using the software RotorGene 4.6 (Corbett Research). The housekeeping gene β-actin was used for normalization.

2.8 Statistics

Values presented in the text are means ± SD. Means of the two groups were compared by Student's *t*-test. Means were considered to be significantly different at $p < 0.05$.

Table 4. Maternal food intake, body and liver weights, and number and weight of pups from dams which received a diet with casein or lupin protein

	Casein diet	Lupin protein diet
Daily food intake of dams (g/day)	31.3 ± 2.9	26.3 ± 1.8[a)]
Body weight of dams, day 7 of lactation (g)	373 ± 20	290 ± 39[a)]
Final body weight of dams, day 18 of lactation (g)	358 ± 17	289 ± 17[a)]
Relative liver weight of dams (g/100 g body weight)	8.3 ± 0.9	10.6 ± 1.1[a)]
Number of pups *per* dam in total	12.9 ± 3.3	12.9 ± 3.6
Number of pups *per* dam alive	12.9 ± 3.3	12.5 ± 3.5
Weight of litters (g)	85.5 ± 17.2	64.0 ± 16.9[a)]
Body weight of pups, at birth (g)	6.76 ± 1.02	5.13 ± 1.07[a)]
Body weight of pups, day 18 of lactation (g)	29.4 ± 5.9	15.6 ± 1.4[a)]

Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group).
a) Different from rats fed casein, $p < 0.05$.

Table 5. Concentration of NEFA in plasma and concentrations of triglycerides and cholesterol in plasma, lipoproteins, liver, and milk of lactating rats which received a diet with casein or lupin protein

	Casein diet	Lupin protein diet
NEFA		
Plasma (mmol/L)	0.95 ±0.27	0.28 ± 0.15[a)]
Triglycerides		
Plasma (mmol/L)	1.11 ± 0.39	0.50 ± 0.13[a)]
Chylomicrons (mmol/L)	0.87 ± 0.35	0.23 ± 0.07[a)]
VLDL (mmol/L)	0.16 ± 0.05	0.24 ± 0.10
Liver (μmol/g)	49.7 ± 13.7	103.5 ± 15.0[a)]
Milk (μmol/g)	105 ± 30	191 ± 65[a)]
Cholesterol		
Plasma (mmol/L)	9.86 ± 4.45	1.88 ± 0.31[a)]
Chylomicrons (mmol/L)	3.19 ± 1.34	0.46 ± 0.05[a)]
VLDL (mmol/L)	4.82 ± 1.39	0.20 ± 0.05[a)]
LDL (mmol/L)	1.59 ± 0.80	0.40 ± 0.07[a)]
HDL (mmol/L)	0.28 ± 0.15	1.11 ± 0.13[a)]
Liver (μmol/g)	97.6 ± 20.5	58.6 ± 6.1[a)]

Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group).
a) Different from rats fed casein, $p < 0.05$.

3 Results

3.1 Food intake, body weight development of the dams, and numbers and body weight of pups

From the 24 rats used in this study, 1 rat of the casein group and 4 rats of the lupin protein group did not conceive. Rats fed the lupin protein diet had a lower food intake than rats fed casein ($p < 0.05$, Table 4). At days 7 and 18 of lactation dams fed the lupin protein diet had a lower body weight than dams fed the casein diet ($p < 0.05$, Table 4). The relative liver weight was higher in dams fed lupin protein than in dams fed casein ($p < 0.05$, Table 4). Litter sizes did not differ between the two groups, whereas the litter weights were lower in the group fed the lupin protein than in the group fed the casein ($p < 0.05$, Table 4). At birth and day 18 of lactation, body weight of the pups from dams fed lupin protein was lower than that of pups from dams fed casein ($p < 0.05$, Table 4).

3.2 Lipid concentrations in plasma, lipoproteins, liver, and milk of the dams

Rats fed lupin protein had lower triglyceride concentrations in plasma and chylomicrons than rats fed casein ($p < 0.05$), whereas the triglyceride concentration in VLDL tended to be higher in the lupin protein group than in the casein group ($p = 0.08$, Table 5). The concentration of triglycerides in liver and milk and the concentration of free fatty acids in plasma were higher in rats fed lupin protein than in rats fed casein ($p < 0.05$, Table 5). The cholesterol concentrations in plasma, chylomicrons, VLDL, LDL, and liver were lower and the concentration of HDL cholesterol was higher in dams fed the lupin protein than in dams fed the casein diet ($p < 0.05$, Table 5).

3.3 Hepatic mRNA concentrations of genes involved in lipid metabolism and LDL receptor concentration

The mRNA abundance of SREBP-1c and the SREBP-1c target genes FAS and SCD-1 in the liver were not different between the two groups of rats (Fig. 1). Rats fed lupin protein had lower mRNA concentrations of PPAR-α and ACO, and higher mRNA concentration of HL in the liver than rats fed casein ($p < 0.05$, Fig. 1). The mRNA abundance of CPT-1a in the liver tended to be lower in dams fed lupin protein than in dams fed casein ($p = 0.061$). The mRNA concentration of the HMG-CoA reductase was significantly higher in rats fed lupin protein than in rats fed casein ($p < 0.05$, Fig. 2). The hepatic mRNA expression of SREBP-2 and LDL receptor tended to be higher in rats fed lupin protein than in rats fed casein ($p = 0.060$ and 0.094, respectively, Fig. 2). Rats fed lupin protein had higher mRNA concentrations of apoA1 and CYP7A1 in the liver than rats fed casein ($p <$

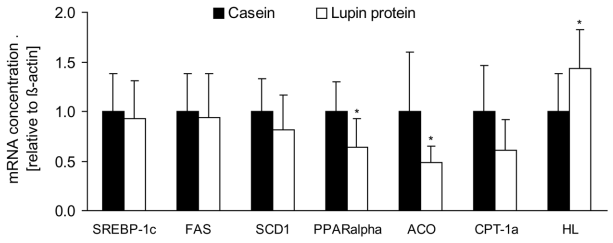

Figure 1. Relative mRNA concentrations of SREBP-1c, FAS, SCD-1, PPAR-α, ACO, CPT-1a, and HL in liver of dams fed either a diet with casein or lupin protein. Total RNA was extracted from rat livers and SREBP-1c, FAS, SCD-1, PPAR-α, ACO, CPT-1a, and HL mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. mRNA concentrations of the genes are shown relative to those of rats fed the casein diet (=1.00). Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group). *Different from rats fed casein, $p < 0.05$.

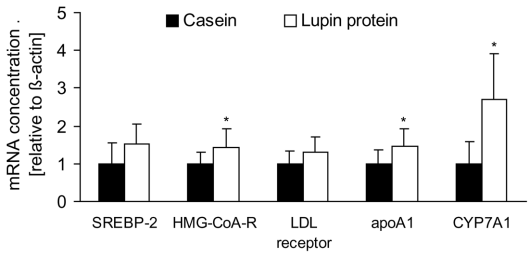

Figure 2. Relative mRNA concentrations of SREBP-2, HMG-CoA reductase, LDL receptor, apo A1, and CYP7A1 in liver of dams fed either a diet with casein or lupin protein. Total RNA was extracted from rat livers and SREBP-2, HMG-CoA reductase, LDL receptor, apoA1, and CYP7A1 mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. mRNA concentrations of the genes are shown relative to those of rats fed the casein diet (=1.00). Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group). *Different from rats fed casein, $p < 0.05$.

0.05, Fig. 2). Livers of rats fed lupin protein had 2.6-fold higher concentrations of LDL receptor than livers of rats fed casein ($p < 0.05$, Fig. 3).

3.4 Relative mRNA concentrations of genes involved in fatty acid synthesis and uptake in the mammary gland

Rats fed lupin protein had higher mRNA concentration of SREBP-1c, FAS, and SCD-1 in the mammary gland than rats fed casein ($p < 0.05$, Fig. 4). The mRNA concentrations of LPL, FABP, FATP, and FAT/CD36 were not different between the two groups of rats (Fig. 4).

4 Discussion

This study aimed to elucidate the effects of lupin protein on lipid metabolism in the lactating organism. In accordance

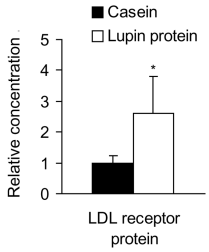

Figure 3. Concentration of LDL receptor protein in liver of dams fed either a diet with casein or lupin protein. The protein concentration of LDL receptor is shown relative to that of rats fed the casein diet (=1.00). Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group). *Different from rats fed casein, $p < 0.05$.

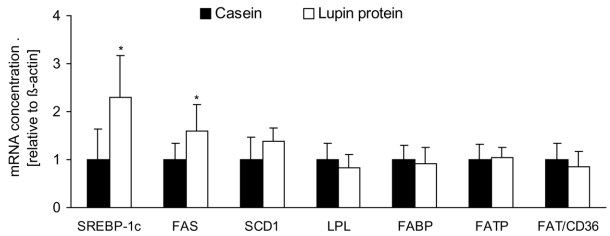

Figure 4. Relative mRNA concentrations of SREBP-1c, FAS, SCD-1, LPL, FABP, FATP, and FAT/CD36 in the mammary gland of dams fed either a diet with casein or lupin protein. Total RNA was extracted from rat mammary glands and SREBP-1c, FAS, SCD-1, LPL, FABP, FATP, and FAT/CD36 mRNA concentrations were determined by real-time detection RT-PCR analysis using β-actin mRNA concentration for normalization. mRNA concentrations of the genes are shown relative to those of rats fed the casein diet (=1.00). Values are means ± SD, $n = 11$ (casein group), $n = 8$ (lupin protein group). *Different from rats fed casein, $p < 0.05$.

to recent studies with nonlactating growing and adult rats [2, 4], lupin protein compared to casein had a hypocholesterolemic effect also in lactating rats, although the reduction of the cholesterol in plasma (−81%), VLDL (−96%), LDL (−75%), and liver (−40%) was much stronger in lactating than in nonlactating rats [2, 4]. We assume that the upregulation of the mRNA and protein levels of LDL receptor was at least in part responsible for the cholesterol-lowering effect of lupin protein. Another factor which might contribute to the cholesterol-lowering effect of lupin protein in plasma and liver was the transcriptional upregulation of CYP7A1. CYP7A1 is the key enzyme of the synthesis of bile acids from cholesterol and provides an important pathway to eliminate abundant cholesterol from the liver [36]. Upregulation of this gene is usually associated with reduced LDL cholesterol concentrations [37, 38].

We further observed that lupin protein compared to casein strongly increased triglyceride concentrations in milk (+81%), liver (+108%), and VLDL (+50%, $p = 0.08$) of lactating rats. These findings are in strong contrast to our recently observed triglyceride-lowering effect of lupin pro-

tein in nonlactating rats [4, 5] suggesting that the effects of lupin protein on lipid metabolism depend on the actual physiological conditions. The observed increase of milk fat content in the group fed lupin protein seems to be caused by a stimulated *de novo* synthesis of lipids in the mammary gland because gene expression of SREBP-1c and the lipogenic enzymes which determine lipogenesis [39, 40] were increased. The high milk fat content appears not to be a result of an increased fatty acid uptake from blood into the mammary gland as the mRNA abundance of genes encoding enzymes and proteins such as LPL, FATP, FAT/CD36, and FABP was not influenced by the lupin protein compared to casein. Milk fat content is known to be influenced by litter size [41] and insufficient intake of indispensable amino acids [42]. To exclude the effects of different litter size and indispensable amino acid deficiencies on milk fat content, we standardized the litters to 10 pups *per* dam and supplemented the diets with methionine and lysine to meet the requirement of all the indispensable amino acids for pregnant and lactating rats [25]. Despite the high milk fat content of dams fed lupin protein, the 18-day-old offspring gained less weight than the offspring of the dams fed casein. Although we could not ascertain the reason for the growth retardation of the pups from the lupin protein-fed dams it is possible that the milk ejection or the milk yield was lower in the lupin protein group than in the casein group. Dams fed lupin protein had a 16% lower food intake than dams fed casein and this could have influenced the milk yield and the lower weight gain of the pups since milk yield is known to depend on food intake [43]. We assume that the remaining alkaloids in the lupin protein are not responsible for the reduced food intake of the dams and the growth retardation of the pups as the alkaloid content of the lupin protein, being 3.2 mg/kg, was relatively low. Indeed, it has been shown that ingestion of *L. angustifolius* with a concentration of 330 mg alkaloids *per* kg has no adverse effect on food intake, weight gain, or other parameters [44].

In contrast to the mammary gland, the increased triglyceride concentration in the liver of dams fed the lupin protein seems not to be caused by changes in lipogenesis as mRNA concentrations of lipogenic enzymes were unchanged. Besides changes in triglyceride synthesis also alterations of fatty acid oxidation in the liver could be responsible for the observed triglyceride accumulation. PPAR-α, a transcription factor belonging to the nuclear hormone receptors, activates fatty acid oxidation by inducing genes involved in fatty acid uptake and fatty acid oxidation [45, 46]. As demonstrated by the downregulation of PPAR-α (–36%) and its target genes ACO (–52%) and CPT-1a (–39%, $p = 0.06$) in the liver of dams fed lupin protein, we assume that the observed triglyceride accumulation in liver and VLDL of these animals was caused by a reduced hepatic fatty acid oxidation.

Another interesting finding from this study concerns the cholesterol concentration of the lipoprotein fraction with a density >1.063 kg/L which we defined as HDL. Dams fed lupin protein compared to dams fed casein had distinctly higher levels of circulating HDL cholesterol. ApoAI is one of the two major protein components of HDL. Upregulation of apoA1 mRNA expression in the liver of dams fed lupin protein may thus provide a possible explanation for increased HDL cholesterol concentrations. Another remarkable finding was that animals which were fed lupin had 74% lower triglyceride and 86% lower cholesterol concentrations in chylomicrons than dams fed casein. The reason for the great differences in chylomicron lipids between the groups is not clear. It is possible, however, that the reduced food intake could be responsible for this finding and could also contribute to the observed reduction of VLDL and LDL cholesterol levels in dams fed the lupin protein diet. Upregulation of hepatic HL, an enzyme that hydrolyzes triglycerides in circulating plasma lipoproteins and facilitates lipoprotein uptake by cell surface receptors [47], provides another possible explanation for the low chylomicron concentrations.

In search of the nutrient factors responsible for the effects of lupin protein on triglyceride and cholesterol metabolism, different protein-associated factors such as amino acids and bioactive peptides generated by hydrolysis of proteins in the intestine could be responsible. Bioactive peptides derived from soybean protein hydrolysates have been reported to have cholesterol-lowering properties due to their stimulating effects on LDL receptor transcription or activity [48 – 50]. Since soy and lupin belong to the same plant family it is highly probable that bioactive peptides from lupines could also be responsible for the effects. A recent study already pointed to the fact that conglutin γ might be one of the cholesterol-lowering peptides in lupin protein [2]. Besides peptides, the amino acid composition of proteins could also contribute to the effects on lipid metabolism observed. Several studies suggested that dietary proteins with high ratios of arginine/lysine and low ratios of methionine/glycine have cholesterol-lowering effect [51 – 53]. In this study, the arginine/lysine ratio of the lupin protein diet was 2.43 compared to 0.43 analyzed in the casein diet. The methionine/glycine ratio was 1.22 in the lupin protein diet and 2.97 in the casein diet. It was speculated that low levels of methionine and high amounts of arginine may act hypocholesterolemic *via* influencing the enterohepatic circulation and the LDL receptors gene expression [53]. Thus, we assume that the hypocholesterolemic effect of lupin protein was in part caused by its amino acid composition.

Taken together, this study demonstrates that lupin protein administered to hypercholesterolemic rats during reproduction lowers the concentrations of circulating cholesterol to normocholesterolemic levels and increases the triglyceride concentrations in plasma, liver, and milk. The effect of lupin protein on triglyceride metabolism in lactating rats is completely different from that of nonlactating rats and

seems to depend strongly on the physiological conditions. Although lupin protein exerts a series of desirable effects on lipid metabolism such as the LDL cholesterol reduction and the HDL cholesterol increase, the triglyceride-raising effect in plasma and liver as well as the growth retardation of the pups are detrimental effects which could possibly limit the use of high amounts of lupin protein during gestation and lactation.

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5 References

[1] Petterson, D. S., in: Gladstones, J. S., Atkins, C. A., Hamblin, J. (Eds.), *Lupins As Crop Plants: Biology, Production, and Utilization*, CAB International, Wallingford, UK 1998, pp. 353 – 384.

[2] Sirtori, C. R., Lovati, M. R., Manzoni, C., Castiglioni, S., *et al.*, Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells, *J. Nutr.* 2004, *134*, 18 – 23.

[3] Marchesi, M., Parolini, C., Diani, E., Rigamonti, E., *et al.*, Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model, *Br. J. Nutr.* 2009, in press; DOI: 10.1017/S000711450894215X.

[4] Bettzieche, A., Brandsch, C., Weiße, K., Hirche, F., *et al.*, Lupin protein influences the expression of hepatic gene involved in fatty acid synthesis and triacylglycerol hydrolysis of adult rats, *Br. J. Nutr.* 2008, *99*, 952 – 962.

[5] Spielmann, J., Shukla, A., Brandsch, C., Hirche, F., *et al.*, Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by reducing hepatic gene expression of sterol regulatory element-binding protein-1c, *Ann. Nutr. Metab.* 2007, *51*, 387 – 392.

[6] Herrera, E., Lipid metabolism in pregnancy and its consequences in the fetus and newborn, *Endocrine* 2002, *19*, 43 – 55.

[7] Herrera, E., Lasunción, M. A., Gomez-Coronado, D., Aranda, P. *et al.*, Role of lipoprotein lipase activity on lipoprotein metabolism and the fate of circulating triglycerides in pregnancy, *Am. J. Obstet. Gynecol.* 1988, *158*, 1575 – 1583.

[8] Williamson, D. H., Regulation of metabolism during lactation in the rat, *Reprod. Nutr. Dev.* 1986, *26*, 597 – 603.

[9] Barber, M. C., Vallance, A. J., Kennedy, H. T., Travers, M. T., Induction of transcripts derived from promoter III of the acetyl-CoA carboxylase-alpha gene in mammary gland is associated with recruitment of SREBP-1 to a region of the proximal promoter defined by a DNase I hypersensitive site, *Biochem. J.* 2003, *375*, 489 – 501.

[10] Scow, R. O., Chernick, S. S., Fleck, T. R., Lipoprotein lipase and uptake of triacylglycerol, cholesterol and phosphatidylcholine from chylomicrons by mammary and adipose tissue of lactating rats *in vivo*, *Biochim. Biophys. Acta* 1977, *25*, 297 – 306.

[11] Smith, J. L., Lear, S. R., Forte, T. M., Ko, W., *et al.*, Effect of pregnancy and lactation on lipoprotein and cholesterol metabolism in the rat, *J. Lipid Res.* 1998, *39*, 2237 – 2249.

[12] Brown, M. S., Goldstein, J. L., The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor, *Cell* 1997, *89*, 331 – 340.

[13] Horton, J. D., Shimomura, I., Sterol regulatory element-binding proteins: Activators of cholesterol and fatty acid biosynthesis, *Curr. Opin. Lipidol.* 1999, *10*, 143 – 150.

[14] Edwards, P. A., Tabor, D., Kast, H. R., Venkateswaran, A., Regulation of gene expression by SREBP and SCAP, *Biochim. Biophys. Acta* 2000, *1529*, 103 – 113.

[15] Sakakura, Y., Shimano, H., Sone, H., Takahashi, A., *et al.*, Sterol regulatory element-binding proteins induce an entire pathway of cholesterol synthesis, *Biochem. Biophys. Res. Commun.* 2001, *286*, 176 – 183.

[16] Vallett, S. M., Sanchez, H. B., Rosenfeld, J. M., Osborne, T. F., A direct role of sterol regulatory element binding protein in activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene, *J. Biol. Chem.* 1996, *271*, 12247 – 12253.

[17] Horton, J. D., Bashmakov, Y., Shimomura, I., Shimano, H., Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice, *Proc. Natl. Acad. Sci. USA* 1998, *95*, 5987 – 5992.

[18] Horton, J. D., Goldstein, J. L., Brown, M. S., SREBPs: Activators of the complete program of cholesterol and fatty acid synthesis in the liver, *J. Clin. Invest.* 2002, *109*, 1125 – 1131.

[19] Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hastry, A. H., *et al.*, Sterol regulatory element-binding protein-1 as a key transcription factor for nutritional induction of lipogenic enzyme genes, *J. Biol. Chem.* 1999, *274*, 35832 – 35839.

[20] Reeves, P. G., Nielsen, F. H., Fahey, G. C., Jr., AIN-93 Purified diets for laboratory rodents: Final report of the American Institute of Nutrition *ad hoc* Writing Committee on the reformulation of the AIN-76A rodent diet, *J. Nutr.* 1993, *123*, 1939 – 1951.

[21] Dabai, F. D., Walker, A. F., Sambrook, I. E., Welch, V. A., *et al.*, Comparative effects on blood lipids and faecal steroids of five legume species incorporated into a semi-purified, hypercholesterolaemic rat diet, *Br. J. Nutr.* 1996, *75*, 557 – 571.

[22] Naveh, E., Werman, M. J., Sabo, E., Neeman, I., Defatted avocado pulp reduces body weight and total hepatic fat but increases plasma cholesterol in male rats fed diets with cholesterol, *J. Nutr.* 2002, *132*, 2015 – 2018.

[23] Yokozawa, T., Ishida, A., Cho, E. J., Nakagawa, T., The effects of Coptidis Rhizoma extract on a hypercholesterolemic animal model, *Phytomedicine* 2003, *10*, 17 – 22.

[24] Yokozawa, T., Cho, E. J., Sasaki, S., Satoh, A., *et al.*, The protective role of Chinese prescription Kangen-karyu extract on diet-induced hypercholesterolemia in rats, *Biol. Pharm. Bull.* 2006, *29*, 760 – 765.

[25] National Research Council, Committee on Animal Nutrition, in: *Nutrient Requirements of Laboratory Animals*, National Academy Press, Washington, DC 1995, pp. 11 – 79.

[26] Sung, J. H., Choi, S. J., Lee, S. W., Park, K. H., *et al.*, Isoflavones found in Korean soybean paste as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, *Biosci. Biotechnol. Biochem.* 2004, *68*, 1051 – 1058.

[27] Bassler, R., Buchholz, H., *Methodenbuch Band III. Die Chemische Untersuchung von Futtermitteln*, VDLUFA-Verlag, Darmstadt 1993.

[28] Schuster, R., Determination of amino acids in biological, pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid chromatography, *J. Chromatogr.* 1988, *431*, 271 – 284.

[29] Fontaine, J., Bech-Andersen, S., Bütikofer, U., De Froidmont-Görtz, I., Determination of tryptophan in feed by HPLC – Development of an optimal hydrolysis and extraction procedure by the EU Commission DG XII in three international collaborative studies, *Agribiol. Res.* 1998, *51*, 97 – 108.

[30] Eder, K., Peganova, S., Kluge, H., Studies on the tryptophan requirement of piglets, *Arch. Tierernahr.* 2001, *55*, 281 – 297.

[31] Hara, A., Radin, N. S., Lipid extraction of tissues with a low toxicity solvent, *Anal. Biochem.* 1978, *90*, 420 – 426.

[32] De Hoff, J. L., Davidson, J. H., Kritchevsky, D., An enzymatic assay for determining free and total cholesterol in tissues, *Clin. Chem.* 1978, *24*, 433 – 435.

[33] Lemieux, C., Gélinas, Y., Lalonde, J., Labrie, F., *et al.*, Hypolipidemic action of the SERM acolbifene is associated with decreased liver MTP and increased SR-BI and LDL receptors, *J. Lipid Res.* 2005, *46*, 1285 – 1294.

[34] Marino, M., Distefano, E., Pallottini, V., Caporali, S., *et al.*, Activation of IP(3)-protein kinase C-alpha signal transduction pathway precedes the changes of plasma cholesterol, hepatic lipid metabolism and induction of low-density lipoprotein receptor expression in 17-beta-oestradiol-treated rats, *Exp. Physiol.* 2001, *86*, 39 – 45.

[35] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 1970, *227*, 680 – 685.

[36] Vlahcevic, Z. R., Pandak, W. M., Stravitz, R. T., Regulation of bile acid biosynthesis, *Gastroenterol. Clin. North Am.* 1999, *28*, 1 – 25.

[37] Shibata, S., Hayakawa, K., Egashira, Y., Sanada, H., Hypocholesterolemic mechanism of Chlorella: Chlorella and its indigestible fraction enhance hepatic cholesterol catabolism through up-regulation of cholesterol 7alpha-hydroxylase in rats, *Biosci. Biotechnol. Biochem.* 2007, *71*, 916 – 925.

[38] Spady, D. K., Cuthbert, J. A., Willard, M. N., Meidell, R. S., Overexpression of cholesterol 7alpha-hydroxylase (CYP7A) in mice lacking the low density lipoprotein (LDL) receptor gene. LDL transport and plasma LDL concentrations are reduced, *J. Biol. Chem.* 1998, *273*, 126 – 132.

[39] Shimano, H., Sterol regulatory element-binding proteins (SREBPs): Transcriptional regulators of lipid synthetic genes, *Prog. Lipid Res.* 2001, *40*, 439 – 452.

[40] Hasty, A. H., Shimano, H., Yahagi, N., Ameniya-Kudo, M., *et al.*, Sterol regulatory element-binding protein-1 is regulated by glucose at the transcription level, *J. Biol. Chem.* 2000, *275*, 31069 – 31077.

[41] Yagil, R., Etzion, Z., Berlyne, G. M., Changes in rat milk quantity and quality due to variations in litter size and high ambient temperature, *Lab. Anim. Sci.* 1976, *26*, 33 – 37.

[42] Crnic, L. S., Chase, H. P., Models of infantile undernutrition in rats: Effects on milk, *J. Nutr.* 1978, *108*, 1755 – 1760.

[43] Flint, D. J., Vernon, R. G., Effects of food restriction on the responses of the mammary gland and adipose tissue to prolactin and growth hormone in the lactating rat, *J. Endocrinol.* 1998, *156*, 299 – 305.

[44] Robbins, M. C., Petterson, D. S., Brantom, P. G. A., 90-day feeding study of the alkaloids of *Lupinus angustifolius* in the rat, *Food Chem. Toxicol.* 1996, *34*, 679 – 686.

[45] Desvergne, B., Wahli, W., Peroxisome proliferator-activated receptors: Nuclear control of metabolism, *Endocr. Rev.* 1999, *20*, 649 – 688.

[46] Hihi, A. K., Michalik, L., Wahli, W., PPARs: Transcriptional effectors of fatty acids and their derivatives, *Cell. Mol. Life Sci.* 2002, *59*, 790 – 798.

[47] Santamarina-Fojo, S., Gonalez-Navarro, H., Freeman, L., Wagner, E. *et al.*, Hepatic lipase, lipoprotein metabolism, and atherogenesis, *Arterioscler. Thromb. Vasc. Biol.* 2004, *24*, 1750 – 1754.

[48] Lovati, M. R., Manzoni, C., Corsini, A., Granata, A., *et al.*, Low density lipoprotein receptor activity is modulated by soybean globulins in cell culture, *J. Nutr.* 1992, *122*, 1971 – 1978.

[49] Manzoni, C., Duranti, M., Eberini, I., Scharnag, H., *et al.*, Subcellular localization of soybean 7S globulin in HepG2 cells and LDL receptor up-regulation by its alpha' constituent subunit, *J. Nutr.* 2003, *133*, 2149 – 2155.

[50] Cho, S. J., Juillerat, M. A., Lee, C. H., Identification of LDL-receptor transcription stimulating peptides from soybean hydrolysate in human hepatocytes, *J. Agric. Food Chem.* 2008, *56*, 4372 – 4376.

[51] Kritchevsky, D., Tepper, S. A., Czarnecki, S. K., Klurfeld, D. M., Atherogenicity of animal and vegetable protein. Influence of the lysine to arginine ratio, *Atherosclerosis*. 1982, *41*, 429 – 431.

[52] Morita, T., Oh-hashi, A., Takei, K., Ikai, M., *et al.*, Cholesterol-lowering effects of soybean, potato and rice proteins depend on their low methionine contents in rats fed a cholesterol-free purified diet, *J. Nutr.* 1997, *127*, 470 – 477.

[53] Gudbrandsen, O. A., Wergedahl, H., Liaset, B., Espe, M., *et al.*, Dietary proteins with high isoflavone content or low methionine – glycine and lysine – arginine ratios are hypocholesterolaemic and lower the plasma homocysteine level in male Zucker fa/fa rats, *Br. J. Nutr.* 2005, *94*, 321 – 330.

