

The α' Subunit from Soybean 7S Globulin Lowers Plasma Lipids and Upregulates Liver β -VLDL Receptors in Rats Fed a Hypercholesterolemic Diet¹

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ABSTRACT Recent data concerning the effect of soybean 7S globulin subunits on the upregulation of LDL receptors in Hep G2 cells identified the α' subunit as the candidate responsible for this biological effect. In vivo evaluation of this subunit on cholesterol homeostasis was hampered by the lack of suitable amounts of α' chain. A novel separation procedure allowed us to investigate the effects of α' subunit administration on plasma cholesterol and triglyceride levels, as well as on the activity of liver β -VLDL receptors of rats fed a hypercholesterolemic (HC) diet. Rats were divided into 9 groups fed the following diets for 28 d: standard diet; HC diet; HC diets + 5, 10, and 20 mg/(kg body weight · d) of α' subunit; HC diets + 50, 100, and 200 mg/(kg body weight · d) of soybean 7S globulin; HC diet + 200 mg/(kg body weight · d) clofibrate. The highest dose of the α' subunit decreased plasma cholesterol and triglycerides 36 and 34%, respectively, in rats fed the HC diet; 10-fold amounts clofibrate reduced plasma cholesterol and triglycerides 38 and 41%. The activity of liver β -VLDL receptors of rats fed the HC diet with the highest dose of the α' subunit had a 96% increase in binding compared with the HC diet group, thus restoring the receptor activity to that of rats fed the standard diet. These results represent the first in vivo evidence of both the plasma lipid-lowering properties and the upregulation of liver β -VLDL receptors induced by the soybean α' subunit. *J. Nutr.* 134: 1334–1339, 2004.

KEY WORDS: • soybean 7S globulin α' subunit • hypercholesterolemic diet • plasma lipids • β -VLDL receptors

Soybeans comprise the most widely grown legume crop in the world. In addition to being an invaluable source of oil and protein for food and feed, soybean proteins have been claimed to be effective in the prevention of cardiovascular diseases (1). Despite intense studies in this area, the protein molecule directly responsible for the observed effects has not been unequivocally identified. Nonetheless, 1 oligomeric soybean 7S globulin, the so-called β -conglycinin, has received more attention in this context because it was shown to have a role in the upregulation of liver high-affinity LDL receptors (2,3). This protein was also shown to reduce plasma triglycerides in humans (4) and in hyperlipidemic rat models (5).

The native 7S globulin is a randomly assorted heterotrimer of α' , α , and β subunits (6), which are the products of a multigene family (7). The relative percentages of α' , α , and β chains in the trimer are ~35, 45, and 20%, respectively (8). All of these subunits are completely N-glycosylated (9). The α' chain is the largest subunit of the 7S soybean globulin oligomer with a M_r of 71 kDa. The other 2 constituent subunits, i.e., the α and β polypeptides, have molecular masses

of 67 and 50 kDa, respectively (10). Assembly of the trimers occurs in the endoplasmic reticulum, the oligomer representing the transport-competent form, which is eventually stored in the storage vacuoles of the soybean cotyledonary cells (10).

In an attempt to identify the biologically active polypeptide(s), Manzoni et al. (11) indirectly demonstrated the putative role of the α' subunit. More recently, the influence of the α' subunit on the increase in LDL uptake and degradation and LDL receptor mRNA levels was shown using Hep G2 cells as a model system (12). That work also reported the interaction of the α' chain, or fragment(s) of it, with 2 proteins involved in fine regulation of cell physiology, namely, thioredoxin 1 and cyclophilin B.

The in vivo evaluation of the effect of this subunit on cholesterol homeostasis has been hampered by the lack of suitable amounts of α' chain; the available methods to separate α' from the other 7S globulin subunits (α and β) have yielded small amounts of purified subunit, suitable only for the in vitro testing. Although the pilot plant isolation of the whole 7S globulin oligomer was described (13), no report is available on the purification of workable amounts of a single 7S globulin subunit because such a preparation involves dissociation of the trimer. Thanh and Shibasaki (14) reported the reversible dissociation of the 3 separate subunits, but the validity of their method was restricted to laboratory scale.

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In this work, we describe the relatively large-scale isolation of soybean 7S globulin α' subunit by a new separation technique based on metal affinity chromatography (MAC) under dissociating conditions. The availability of this isolated polypeptide chain allowed us to investigate the effect of daily administration of the α' subunit on plasma cholesterol and triglyceride levels as well as on the activity of liver β -VLDL receptors in rats fed a hypercholesterolemic (HC) diet.

MATERIALS AND METHODS

Reagents. Cold defatted soybean flakes, MAC resin (iminodiacetic acid agarose), clofibrate, carboxymethyl cellulose, and albumin serum were purchased from Sigma-Aldrich. Kits for the enzymatic assay of cholesterol and triglycerides were A11A00051 and A11A00069, respectively, both from ABX Diagnostics (Horiba group). The Protein Coomassie Plus Protein Assay kit was purchased from Pierce. 125 Iodine, carrier free, in 100 mmol/L NaOH, was from Perkin Elmer Life Sciences. Sephadex G25 columns (PD10) were from Pharmacia Biosciences. Acrylamide monomers and catalysts, the protein II vertical electrophoresis chamber and the PowerPac 300 power supply were from BioRad. Studioscan II Scanner was from Agfa. All other chemicals were of analytical grade and were from Merck.

Isolation of soybean 7S globulin. For the isolation of the 7S globulin, a modification of a published procedure (13) was used. Our procedure consisted in the selective extraction of the 7S globulin with 7 mmol/L NaHSO_3 at 4°C with stirring for 16 h. The flake weight to buffer volume ratio was 1:15. Centrifugation at $8000 \times g$ for 1 h at the same temperature allowed the recovery of a supernatant, which consisted essentially of 7S globulin, and a pellet containing all insoluble materials, including the glycinin fraction and a residual amount of 7S globulin. The 7S globulin was recovered from the supernatant by 40% aqueous ethanol precipitation and centrifugation as above. The pellet was then freeze-dried.

Isolation of the α' subunit. Urea (8 mol/L) in 50 mmol/L Tris-HCl, pH 7.4, containing 0.5 mol/L NaCl (loading buffer) was used as a denaturing agent to dissociate the 7S globulin subunits. The resulting solution was applied to a MAC matrix coupled with Zn^{2+} , in a ratio of 30 g of 7S globulin/L of resin. The resin was thoroughly washed with the loading buffer to elute the unbound fraction. The bound fraction was subsequently eluted with 0.1 mol/L imidazole in the loading buffer. The recovered 7S globulin α' subunit was then precipitated and washed with 40% aqueous ethanol before freeze-drying. This procedure was applied to 250 g of defatted soybean flakes to obtain 5 g of α' subunit. The whole procedure, which is outlined in Figure 1, has been patented (15).

Total soybean proteins for SDS-PAGE analysis were directly extracted with sample denaturing buffer containing 3.75% SDS and 350 mmol/L 2-mercaptoethanol. Protein concentrations were determined with the Bradford method (16), using bovine serum albumin as the standard protein.

Electrophoretic techniques. SDS-PAGE was carried out on 12% polyacrylamide gels, using a Minigel Bio-Rad system, as described by Laemmli (17). Gels were stained with Coomassie Brilliant Blue R250. Gels were digitized by the Studioscan II Scanner and processed with Image Master 1D Elite, version 4.00 (Nonlinear Dynamics, Amersham Pharmacia Biotech) for band quantification.

Animals, diets and experimental protocol. Male Sprague-Dawley rats (Charles River Italia; body weights 130–150 g) were housed in a room with controlled lighting (12 h/d), constant temperature (18°C) and relative humidity (55–65%). During wk 1, they were fed a commercial nonpurified diet (standard diet; Mucedola S.r.l., Settimo Milanese) and then divided into 9 groups ($n = 10/\text{group}$) on the basis of the plasma cholesterol concentrations and body weight, so that the distribution among the groups was similar. One group continued to consume the standard diet; the other rats were transferred to a Nath's diet, a well-characterized and validated HC regimen for rats (1.0 g/100 g cholesterol and 0.5 g/100 g cholic acid) (18). Feed and water were freely available. Isolated α' subunit [doses: 5, 10, 20 mg/(kg body weight)] and soybean 7S globulin [doses: 50, 100, 200 mg/(kg body weight)], dissolved in 5 g/L carboxymethylcellulose were

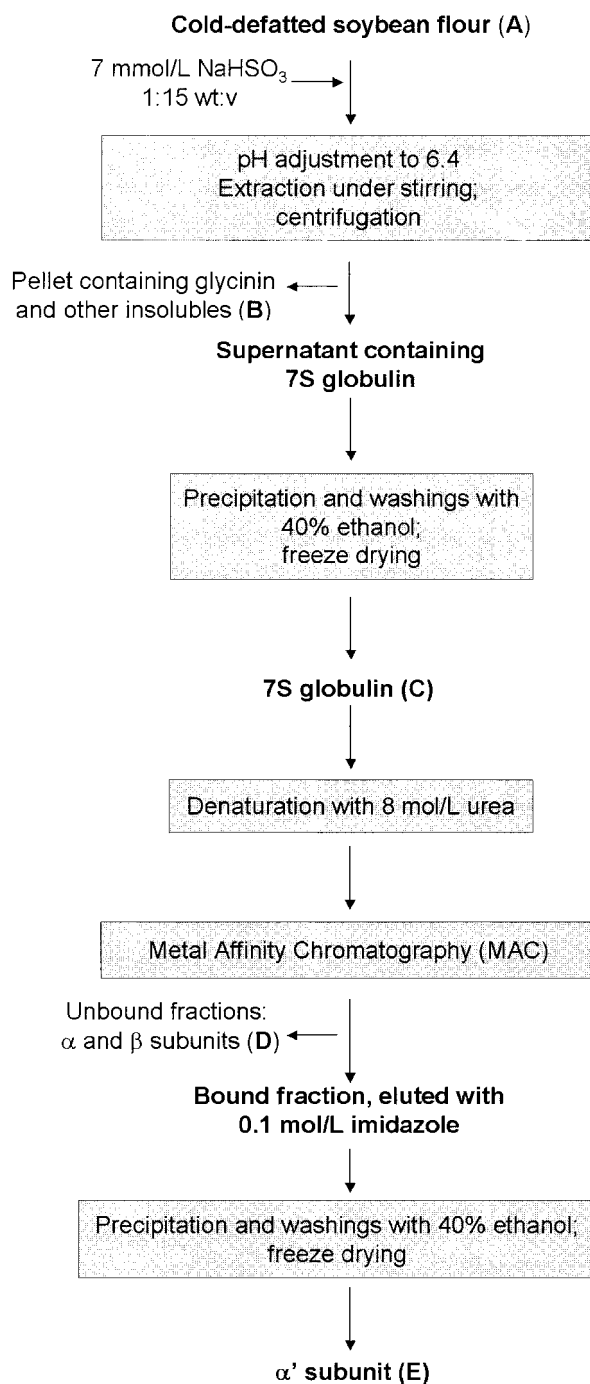


FIGURE 1 The purification steps of the α' subunit from soybean flour. Capital letters between parentheses refer to the electrophoretic patterns of Figure 2.

administered daily by gavage at 0900 h; the vehicle alone was administered to rats fed the HC and standard diets. Clofibrate, a lipid-lowering drug (19), was used at 200 mg/(kg body weight) as a reference drug and administered to 1 group of rats. Therefore, the groups were as follows: standard diet; HC diet (control group); HC diets + 5, 10, and 20 mg/(kg body weight \cdot d) of α' chain, respectively; HC diets + 50, 100, and 200 mg/(kg body weight \cdot d) of soybean 7S globulin, respectively; and HC diet + 200 mg/(kg body weight \cdot d) clofibrate. The experiment lasted 28 d; body weight gain and food intake were recorded for each group weekly. Blood samples, collected in the presence of EDTA (1 g/L), were obtained from rats that had been deprived of food for 18 h and killed by guillotine while

under diethyl ether anesthesia; livers were removed for further analyses, as described below. Plasma total cholesterol and triglycerides were determined by enzymatic methods (ABX Diagnostics).

VLDL with β -mobility (β -VLDL, $d < 1.019$ kg/L) for the binding studies were obtained from male New Zealand rabbits (Harlan Italy) that had been deprived of food for 18 h after consuming a hypercholesterolemic diet (2.0 g/100 g cholesterol, 20 g/100 g casein, 15 g/100 g SFA; Piccioni) for 28 d (20). Proteins were labeled with Na^{125}I , as previously described (2).

All procedures involving rats and rabbits and their care were performed according to the Italian Government Guidelines for animal tests and in agreement with the E.C. rules (86/609/EEC) and supervised by the Laboratory Animal Welfare Service of our Department.

Preparation of liver membranes and receptor studies. The binding parameters of labeled β -VLDL to liver receptors were evaluated by an established model (21). Liver membranes from rats fed the standard diet, HC diet, and HC diet including 20 mg/(kg body weight \cdot d) of α' subunit were prepared according to Kovanen et al. (22). Livers were removed and placed in ice-cold 150 mmol/L NaCl solution immediately after the rats were killed and processed as described earlier (23). Binding studies were carried out in quadruplicate with pools of membranes, obtained from all rats in each experimental group. Membrane proteins (70–100 μg) and 25–550 ng of ^{125}I -labeled β -VLDL were incubated in the absence or presence of excess (30 μg /sample) unlabeled β -VLDL. Specific binding was calculated by subtracting the amount of ^{125}I -labeled β -VLDL bound in the presence of excess unlabeled β -VLDL from that bound in its absence, as previously described (23). The equilibrium dissociation constants (K_d) and maximal amounts of bound lipoproteins (B_{max}) were calculated by Scatchard analysis.

Statistical analyses. Statistical analyses of the individual differences in plasma lipids between rats fed the standard diet and rats fed the HC diet (control group) were evaluated with an unpaired Student's t test. In the case of repeated comparisons (rats fed the HC diet alone vs. the HC diet + different amounts of soybean proteins or clofibrate, i.e., the 8 groups), differences in plasma lipids were evaluated by ANOVA followed by Dunnett's post-hoc test; in this analysis, the rats fed the HC diet alone were defined as the control group. Statistical analyses of the β -VLDL data were also carried out by ANOVA followed by Dunnett's post-hoc test, but in this case, the control group was rats fed the standard diet. The lipoprotein binding curves were analyzed according to Munson and Rodbard (24). Values are expressed as means \pm SEM. Differences with P -values < 0.05 were considered to be significant.

RESULTS

Isolation of the soybean 7S globulin α' subunit. The selective extraction of 7S globulin from soybean flakes was adapted from a previous procedure (13) to improve yield and purity of this protein fraction. Specifically, a lower temperature and longer extraction times were used, thus increasing the insolubility of the 11S globulin fraction. The subsequent precipitation and washing steps with aqueous ethanol of the 7S globulin fraction were found to help, both by removing low-molecular-weight compounds and salts and by improving subsequent freeze-drying. This step was especially relevant during the scaling-up of the procedure to obtain a purified α' subunit suitable for *in vivo* testing. The isolation of the α' chain, which is a subunit of the soybean 7S globulin trimer, implied a denaturation step to dissociate the oligomer. This was achieved by incubating the protein in the MAC loading buffer containing 8 mol/L urea at 50°C for 1 h. This step was shown to completely dissociate the oligomer (25). Actual purification of the α' subunit was achieved by the Zn^{2+} -coupled MAC step (Fig. 1). This procedure was adopted on the strength of the observation that the α' subunit contains a histidine-rich N-terminal extension with respect to the α subunit. This region is missing in the β subunit. Histidine residues in polypeptides

are widely used as purification molecular tools to bind various transition metal ions, including Ni^{2+} and Zn^{2+} under both native and denaturing conditions. In addition, a greater number of glutamate residues are also present in the α' subunit extension region compared with the α subunit. Indeed, when denatured 7S globulin was loaded onto the column, a large peak of unretained protein material was obtained (not shown) and subsequently identified by SDS-PAGE to contain predominantly the α and β subunits (Fig. 2, lane D). The fraction retained by the Zn^{2+} -coupled matrix and eluted with imidazole consisted essentially of the α' subunit (Fig. 2, lane E) and was thus considered suitable for rat testing. The overall yields of α' subunit starting from the 7S globulin and from defatted soybean flakes were 13 and 2% by weight, respectively. These yields did not change when the procedure was applied to 250 g of soybean flakes.

Effect of the 7S globulin and its α' subunit on plasma lipids and hepatic β -VLDL receptor activity. At the end of the 28-d treatment, the weight gains of the 9 groups of rats were not different (data not shown). Plasma lipid levels in rats fed the HC diet differed from those of rats fed the standard diet ($P < 0.001$, Fig. 3). Rats administered the 2 highest doses of α' subunit and 7S globulin had significantly lower plasma cholesterol and triglyceride concentrations than the control group fed the HC diet. In rats administered 20 mg/(kg body weight \cdot d) of the purified α' subunit, plasma cholesterol and triglyceride levels were decreased 36 and 34%, respectively, and 200 mg/(kg body weight \cdot d) of 7S globulin resulted in decreases of 49 and 50%, respectively. These latter results likely reflected the greater amount of α' subunit present in the 200 mg/(kg body weight \cdot d) dose of 7S globulin. The cholesterol and triglyceride levels in the group treated with 200 mg/(kg body weight \cdot d) clofibrate were decreased 38 and 41%, respectively.

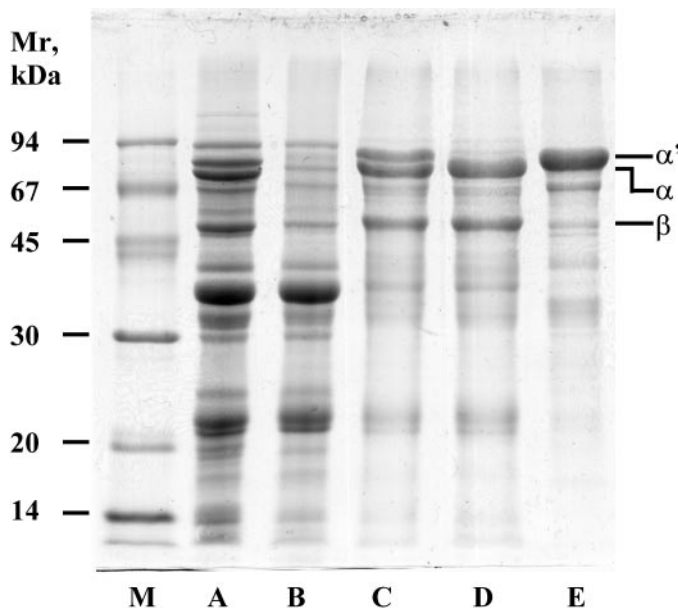


FIGURE 2 SDS-PAGE under reducing conditions of the 7S globulin and its subunits separated according to the purification procedure described in the Methods and outlined in Figure 1. Lane A: soybean total protein extract; Lane B: 11S globulin (glycinin); Lane C: 7S globulin (β -conglycinin); Lane D: MAC unretained fraction, consisting essentially of α and β subunits; Lane E: MAC bound fraction, consisting essentially of α' subunit; Lane M: marker proteins. Sample amounts ranged from 4 to 20 μg .

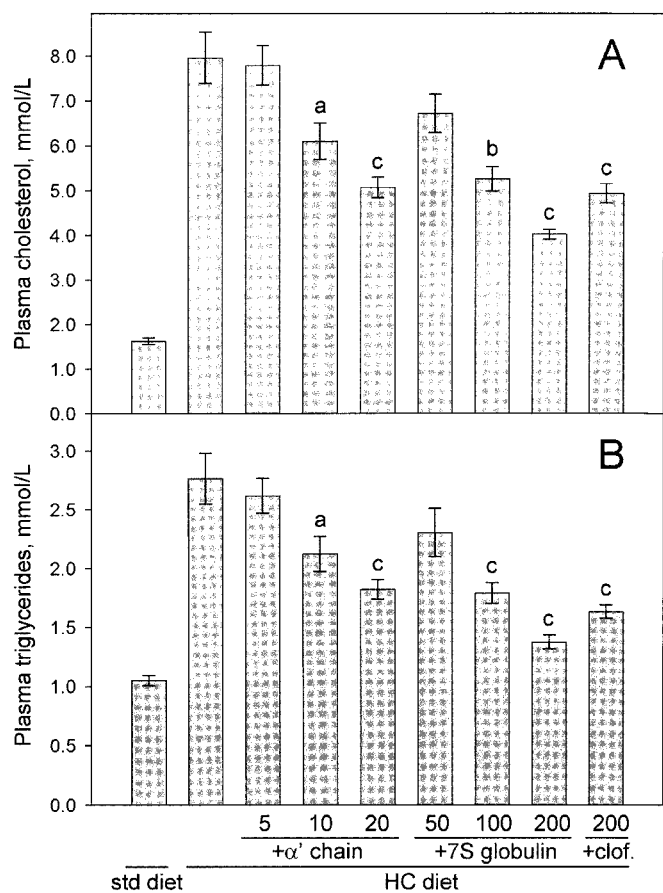


FIGURE 3 Plasma total cholesterol (A) and triglyceride (B) concentrations of rats fed a HC diet for 28 d with and without different doses of soybean α' subunit, 7S globulin, and clofibrate (clof.). Doses (5–20; 50–200; 200) are mg product administered/(kg body weight \cdot d). The 3 doses of 7S globulin contained α' subunit doses of \sim 17, 34, and 68 mg/(kg body weight \cdot d) (8). Values for rats fed a standard diet are also reported (std diet). Values are means \pm SEM, $n = 10$ rats. Letters indicate differences from the HC diet mean: ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

Rats fed the HC diet had fewer liver membrane binding sites than rats fed the standard diet (Table 1), as already shown by our group in ex vivo (21) and in vivo models (27). Maximal binding was, in fact, 43% lower in these rats compared with rats fed the standard diet ($P < 0.005$) (Fig. 4). In rats fed the HC diet and administered the α' subunit, the B_{\max} for labeled β -VLDL was 96% greater than in the HC-fed rats (Table 1); the α' subunit oral administration thus restores the receptor activity, normally depressed by HC diet because the β -VLDL specific binding did not differ significantly from that of rats fed the standard diet. The Scatchard plot quantitative analyses, by allowing the estimation of the K_d (Table 1), suggest that lipoproteins are bound to similar receptor sites under all test conditions.

DISCUSSION

Employing a soybean protein-containing dietary intervention for the management of lipid disorders was recommended recently by the Adult Treatment Panel III of the USA National Cholesterol Education Program (28), and by the American Heart Association (29); the cholesterol-lowering effect has been shown to act through activation of the LDL receptor

pathway (2,30). Moreover, Jenkins et al. (31) demonstrated that a dietary portfolio, consisting in a diet low in SFA, containing soybean proteins, nuts, plant sterols, and viscous fibers, administered to healthy hyperlipidemic subjects, was as effective in lowering cholesterol and C-reactive protein as was statin treatment. From these data, Anderson (32) suggested that a prudent diet, containing soybean proteins, may be a potent tool to achieve plasma lipid reduction, particularly in those patients who are nonresponsive or cannot tolerate drug therapy.

Our group is particularly active in studying the biological consequence of soybean protein intake both in hypercholesterolemic patients (33) and in validated rat models (21,23). Recently, we showed that the α' subunit from soybean 7S globulin plays a pivotal role in the cholesterol homeostasis of Hep G2 cells, a human hepatoma cell line, after detection of an increase in LDL receptor mRNA levels (12).

The preparative isolation of the 7S soybean globulin α' subunit, described in this paper, allowed us for the first time to test in vivo the effect of this polypeptide on plasma lipid homeostasis in a validated model of hypercholesterolemia. In fact, although our previous in vitro data indicated that the α' subunit was responsible for the upregulation of LDL receptors (11,12), the lack of a separation procedure that could provide sufficient amounts of purified α' chain hampered us from testing in vivo the effect of this subunit on both plasma lipid levels and hepatic β -VLDL receptors. In this work, the isolated α' chain, although not pure to homogeneity, was at least 5-fold enriched compared with its content in the 7S globulin fraction (data not shown) and was considered suitable for in vivo testing.

The oral administration of both soybean 7S globulin and the α' chain thereof to hypercholesterolemic rats significantly reduced plasma cholesterol and triglyceride levels. These data represent the first in vivo confirmation that the α' subunit is responsible for the biological activity already described in vitro (3). Moreover, the data obtained in the present experiment on the plasma lipid-lowering effect of an oral administration of the soybean 7S globulin to HC rats confirm our previous

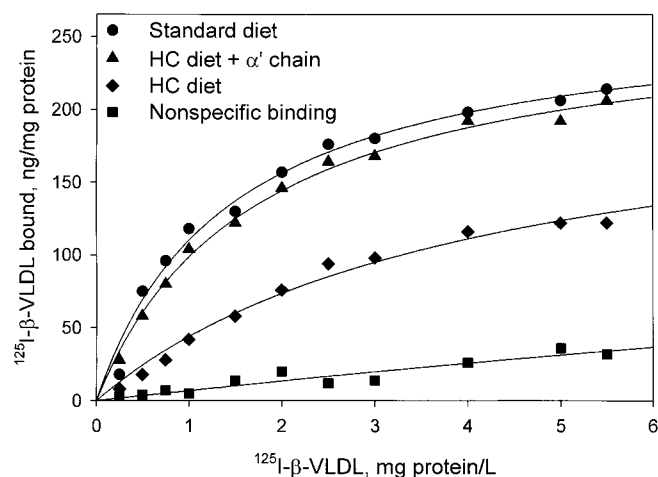


FIGURE 4 Saturation binding curve of ^{125}I - β -VLDL, from donor rabbits fed an HC diet, to liver membranes of rats fed the standard diet or the HC diet and administered the purified α' subunit at 0 or 20 mg/(kg body weight \cdot d). The data represent specific binding, calculated by subtracting the amount of ^{125}I bound in the presence of an excess unlabeled β -VLDL (nonspecific binding) from that bound in its absence (curves not shown). Each point represents the mean of 4 separate experiments.

TABLE 1

Characteristics of β -VLDL binding to liver membrane preparations from rats fed standard, HC, and HC + α' subunit diets¹

	Standard diet	HC diet	HC diet + α' subunit ²
Equilibrium dissociation constant (K_d), mg protein/L	1.13 \pm 0.36	1.08 \pm 0.25	1.18 \pm 0.20
Amount of lipoprotein bound at saturation (B_{max}), ng protein/mg membrane protein	303.8 \pm 35.2	174.2 \pm 23.3 ^a	341.0 \pm 29.3
Correlation coefficient (r) ³	0.95	0.89	0.98

¹ Values are means \pm SEM, $n = 4$. ^a Different from standard diet, $P < 0.005$.

² The α' subunit was 20 mg/(kg body weight \cdot d).

³ Coefficient r is derived from the Scatchard plots of data from Figure 4.

observations achieved in the same in vivo experimental model (2), in which the treatment lasted only 2 wk because at that time, this new procedure for the scaling-up of the soybean globulins and the subunits thereof was not available.

A related concern is the biological fate of the α' subunit in vivo because it seems unlikely that it crosses the intestinal barrier with no modification. We hypothesized, in fact, that peptides, deriving from the activity of gastric/intestinal enzymes on soybean proteins, might be absorbed by the enterocytes and reach the liver through the blood stream, where they elicit the biological effect, as discussed previously (3,12,34). This aspect, as well as the molecular properties of the isolated α' subunit, including its susceptibility to proteolysis, is outside the scope of this work and is currently being investigated by our group.

The results of the present study, in our opinion, are extremely intriguing because they show for the first time that a dietary protein is active at concentrations that are lower than those reported for hypolipidemic drugs. This work also suggests that biologically active peptides, capable of modulating cholesterol homeostasis through LDL receptor upregulation, are likely produced in vivo. Indeed, a marked increase in β -VLDL receptors was detected in hypercholesterolemic rats treated with the α' subunit. Moreover, in the present study, a significant decrease in plasma triglycerides was detected after administration of the α' subunit and 7S globulin. The mechanism whereby soybean proteins lower plasma triglycerides in vivo might be due to decreased lipid synthesis and apolipoprotein B secretion, as demonstrated in Hep G2 cells exposed to 7S soybean globulin (34). Recently, a triglyceride-lowering effect was demonstrated by Kambara et al. (4) in a small group of patients consuming daily 2 crackers containing soybean 7S globulin. The finding in this work that the α' subunit is responsible for the lipid-lowering effects of soybean protein explains the previously observed effects of the 7S globulin. On the basis of the estimated amounts of α' subunit in the 7S globulin trimer, i.e., around one third of the total globulin weight (8), it will now be possible to calibrate the doses administered in terms of the actual active component. Our data also showed that it is possible to enhance the lipid-lowering effects by further increasing the α' subunit doses.

In conclusion, this study provides the first direct in vivo evidence of both the plasma lipid-lowering effect of the α' subunit from soybean 7S globulin, and the upregulation of the β -VLDL receptors in liver cells from hypercholesterolemic rats in response to oral treatment with this polypeptide. Studies are in progress to identify the polypeptide region(s) responsible for this activity, thus opening the way to a detailed analysis of the metabolic fate of this soybean subunit and to the eventual elucidation of the mechanism(s) underlying the observed effects. Moreover, because the α' subunit proved to be biologically active at relatively low doses, our ongoing investigations

will evaluate the efficacy in humans of this promising polypeptide in view of its potential utilization as a nutraceutical, alone or combined with drugs, in lipid-lowering therapy.

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